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Molecular diversity of *bla* genes in *Klebsiella pneumoniae* and *Escherichia coli* isolates

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Abstract

Antimicrobial resistance is a growing problem worldwide, with extended-spectrum β-lactamase (ESBL)-producing organisms remaining an important cause of cephalosporin therapy failure. The main purpose of the work presented in this dissertation was to search for the molecular diversity of Ambler class A β-lactamase encoding genes in clinical *Klebsiella pneumoniae* and *Escherichia coli* isolates and its consequences.

In a first step, the evaluation of ESBL detection and confirmation methods for *K. pneumoniae*, using different guidelines, was performed, with nucleotide sequencing allowing the identification of a new ESBL (SHV-55). The enzymatic properties of this new β-lactamase confirmed a higher affinity towards extended-spectrum cephalosporins, which is characteristic of ESBLs, contrasting to the parental enzyme SHV-1.

For the new β-lactamase SHV-72, the higher value of 50% inhibitory concentration of clavulanic acid than for SHV-1 correlated with the values of higher afinity towards penicillins, which are characteristic of an inhibitor resistance enzyme. Simulation of molecular dynamics suggested that the Lys234Arg substitution in SHV-72 was responsible for stabilizing an atipical conformation of the Ser130 side chain, which may decrease susceptibility to clavulanic acid by preventing cross-linking between Ser130 and Ser70.

Among the collection of *K. pneumoniae* strains studied, we identified several genes coding for different enzymes belonging to the CTX-M, GES, LEN, OKP, OXA, TEM, and SHV families and, among them, 35 were new β-lactamases. Among ESBL-producing isolates from 1999 and 2006, we detected the presence of CTX-M enzymes only in the latest period.

Overall, the complex molecular diversity of the bla_{SHV} genes detected impelled us to propose a classification for this gene family, based on nucleotide synonymous mutations and the presence or absence of the nonsynonymous mutation T92A.

Finally, among *E. coli* isolates colected in Portugal, a predominat multidrug resistant clone, producing TEM, OXA and CTX-M enzymes, was present in different hospital wards and community environments. The high dissemination of the CTX-M enzymes detected could also be associated with horizontal transfer of plasmids or mobile elements, like IS*Ecp1*. In Algeria, we identified the same insertion sequence in clinical *E. coli* strains producing TEM and either CTX-M-3 or CTX-M-15 β-lactamases.

In conclusion, the results from this dissertation extended our knowledge about the most important mechanism of resistance – the β-lactamases production – focusing on the phenotypic characteristics, biochemical properties, structure-function relationships, molecular diversity and dissemination, which are of most importance to the comprehension of the general resistance scenario in Portugal and worldwide.

Keywords: *Klebsiella pneumoniae*; *Escherichia coli*; molecular diversity; dissemination; Portugal; β-lactamases; antimicrobial resistance

Resumo

A resistência aos antimicrobianos é um problema crescente a nível mundial, sendo os organismos produtores de β-lactamases de espectro alargado (extended-spectrum β-lactamase, ESBL), uma causa importante da falência terapêutica de cefalosporinas. A presente dissertação teve como principal objectivo investigar sobre diversidade molecular dos genes que codificam β-lactamases da classe A de Ambler em isolados clínicos de *Klebsiella pneumoniae* e *Escherichia coli* e avalias sobre as suas consequências.

Numa primeira fase, foram avaliados métodos de detecção e confirmação de ESBLs em *K. pneumoniae,* utilizando diferentes normas, onde a sequenciação nucleotídica permitiu a identificação de uma nova ESBL (SHV-55). As propriedades enzimáticas desta β-lactamase evidenciaram a sua maior afinidade para as cefalosporinas de espectro alargado, característico das ESBLs, em contraste com a enzima parental SHV-1.

A nova β-lactamase SHV-72, apresentou um valor superior de concentração de ácido clavulânico do que SHV-1 para a inibição de 50% da actividade enzimática, estando este de acordo com os valores superiores de afinidade que também evidencia para as penicilinas, sendo estes caracteristicos das enzimas resistentes aos inibidores. Simulações de dinâmica molecular sugeriram que a substituição aminoacídica Lys234Arg presente em SHV-72 era responsável pela estabilização da conformação da cadeia lateral da Ser130, impedindo a ligação entre esta e a Ser70, o que poderá diminuir a susceptibilidade ao ácido clavulânico.

Na colecção de isolados de *K. pneumoniae* estudados, foram identificados vários genes que codificam diferentes enzimas pertencentes às famílias CTX-M, GES, LEN, OKP, OXA, TEM, e SHV, das quais 35 eram novas β-lactamases. Entre os isolados produtores de ESBLs de 1999 e de 2006, apenas foi evidenciada a presença de enzimas CTX-M no último período.

A complexa diversidade molecular detectada nos genes bla_{SHV}, levou-nos a propor uma classificação para esta família de genes, baseada nas mutações nucleotídicas sinónimas e na presença ou ausência da mutação não-sinónima T92A.

Finalmente, entre os isolados de *E. coli* colectados em Portugal, foi detectado um clone multirresistente predominante, em diferentes serviços hospitalares e comunidade, produtor de enzimas TEM, OXA e CTX-M. A elevada disseminação de enzimas CTX-M detectada poderá estar igualmente associada à transferência horizontal de plasmídeos ou de elementos móveis, como IS*Ecp1*. Na Algéria foi identificada a mesma sequência de inserção em isolados de *E. coli* produtores de β-lactamases TEM e CTX-M-3 ou TEM e CTX-M-15.

Em conclusão, os resultados apresentados na presente dissertação ampliam o nosso conhecimento sobre o mais importante mecanismo de resistência – a produção de β-lactamases – evidenciando características fenotípicas, propriedades bioquímicas, relação estrutura-função, diversidade molecular e disseminação, as quais são da maior importância para a compreensão da resistência em Portugal e no mundo.

Palavras-Chave: *Klebsiella pneumoniae*; *Escherichia coli*; diversidade molecular; disseminação; Portugal; β-lactamases; resistência aos antimicrobianos.

Thesis Outline

β-Lactamase-producing *Enterobacteriaceae* are a growing concern in human medicine today. The studies presented in this PhD dissertation are focused on the molecular diversity of *bla* genes in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from community and hospital environments, and its consequences at the biochemical and epidemiological levels.

The order of presentation of each chapter in the present PhD dissertation does not necessarily reflect a chronological order, since some of the works described below were done simultaneously and the results obtained during one particular work would influence the progress of the other and vice-versa.

Chapter I briefly describes the current knowledge of β-lactam resistance mechanisms. Special attention was given to the mode of action of β-lactamases, their structure and properties, the resistance they confer and distribution of different families.

Chapter II describes the occurrence of a novel SHV-type enzyme (SHV-55) in isolates of *K. pneumoniae* in a comparitive study for the evaluation of the production of extended-spectrum β-lactamases.

Chapter III reports the biochemical characterization of SHV-55, an extended-spectrum class A β-lactamase.

Chapter IV describes the role of the Lys234Arg substitution in SHV-72 as a determinant for resistance to inhibition by clavulanic acid; this substitution is essential to better comprehend the functionality of the serine β-lactamase active site.

Chapter V presents the molecular epidemiology and antimicrobial susceptibility of extended- and broad-spectrum β-lactamase-producing *K. pneumoniae* isolates in Portugal.

Chapter VI describes the detection and characterization of OKP and LEN β-lactamases produced by clinical *K. pneumoniae* strains in this country.

Chapter VII reports the high diversity of bla_{SHV} genes based on nucleotide synonymous mutations and the presence or absence of the nonsynonymous mutation T92A, which led to the proposal of a classification.

Chapter VIII describes, for the first time in Portugal, the co-expression of CTX-M-15, OXA-30 and TEM-1 β-lacatamases in clinical *E. coli* isolates.

Chapter IX reports the dissemination of extended-spectrum β-lactamase CTX-M-producing clinical *E. coli* isolates in community and nosocomial environments in Portugal.

Chapter X reports the identification of CTX-M-3 and CTX-M-15 extended-spectrum β-lactamases in isolates of *E. coli* from a hospital in Algiers, Algeria.

Chapter XI presents an overall discussion of the results described in this PhD dissertation, as well as several questions raised along the course of this work.

Chapters II to X may be read separately. They contain reproductions of the following publications:

Mendonça N, Ferreira E & Caniça M (2006) Occurrence of a novel SHV-type enzyme (SHV-55) among isolates of *Klebsiella pneumoniae* from Portuguese origin in a comparison study for extended-spectrum β-lactamase-producing evaluation. *Diagn Microbiol Infect Dis* **56**: 415−420. (Chapter II).

Mendonça N, Manageiro V, Bonnet R & Caniça M (2008) Biochemical characterization of SHV-55, an extended-spectrum class A β-lactamase from *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* **52**: 1897−1898. (Chapter III).

Mendonça N, Manageiro V, Robin F, Salgado MJ, Ferreira E, Caniça M & Bonnet R (2008) The Lys234Arg substitution in the enzyme SHV-72 is a determinant for resistance to clavulanic acid inhibition. *Antimicrob Agents Chemother* **52**: 1806−1811. (Chapter IV).

Mendonça N, Ferreira E, Louro D, ARSIP Participants & Caniça M (2008) Molecular epidemiology and antimicrobial susceptibility of extended- and broad-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolated in Portugal. *Int J Antimicrob Agents* doi:10.1016/j.ijantimicag.2008.11.014 (Chapter V).

Mendonça N, Ferreira E, Antibiotic Resistance Surveillance Program in Portugal (ARSIP) & Caniça M (2008) OKP and LEN β-lactamases produced by clinical *Klebsiella pneumoniae* strains in Portugal. *Diagn Microbiol Infect Dis* **63**: 334-338 (Chapter VI).

Mendonça N, Nicolas-Chanoine MH & Caniça M (2008) Diversity of the bla_{SHV} genes. Submitted to *Diagn Microbiol Infect Dis*. (Chapter VII).

Mendonça N, Louro D, Castro AP, Diogo J & Caniça M (2006) CTX-M-15, OXA-30 and TEM-1-producing *Escherichia coli* in two Portuguese regions. *J Antimicrob Chemother* **57**: 1014−1016. (Chapter VIII).

Mendonça N, Leitão J, Manageiro V, Ferreira E, Antimicrobial Resistance Surveillance Program in Portugal & Caniça M (2007) Spread of extended-spectrum β-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. *Antimicrob Agents Chemother* **51**: 1946−1955. (Chapter IX).

Ramdani-Bouguessa N, Mendonça N, Leitão J, Ferreira E, Tazir M & Caniça M (2006) CTX-M-3 and CTX-M-15 extended-spectrum β-lactamases in isolates of *Escherichia coli* from a hospital in Algiers, Algeria. *J Clin Microbiol* **44**: 4584−4586. (Chapter X).

Abreviations

General Abreviations

ARSIP	Antibiotic Resistance Surveillance Program in Portugal
bla	β-Lactamase coding gene
BSAC	British Society for Antimicrobial Chemotherapy
CLSI	Clinical and Laboratory Standards Institute
CR	Common Region
CRG	Commissie Richtlijnen Gevoeligheidsbepalingen
DAP	Diaminopimelic Acid
DIN	Deutsches Institut für Normung
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended-Spectrum β-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
IC_{50}	Fifty Percent Inhibitory Concentration
IEF	Isoelectric Focusing
IS	Insertion Sequence
IS*CR*	Insertion Sequence Common Region
k_{cat}	Catalytic Activity Constant
K_m	Michaelis Constant
KpI	*Klebsiella pneumoniae* group I
KpII	*Klebsiella pneumoniae* group II
KpIII	*Klebsiella pneumoniae* group III
MIC	Minimal Inhibitory Concentration
MDSs	Molecular Dynamic Simulations
NAG	N-acetylglucosamine
NAM	N-acetylmuramic
NCCLS	National Committe for Clinical Laboratory Standards
NIH	National Institute of Health
NWGA	Norwegian Working Group on Antibiotics
Omp	Porine, “Outer Membrane Protein”
ORF	Open Reading Frame
PBP	Penicillin-Binding Protein
pCMB	p-Chloromercuribenzoate
PCR	Polymerase Chain Reaction

PCR-RFLP	Restriction Fragment Length Polymorphism - Polymerase Chain Reaction
PCR-SSCP	Single Strand Conformation Polymorphism - Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RLC	Reference Laboratory Criteria
RND	Resistance Nodulation Division
RMSD	Root Mean Squared Deviation
SFM	Société Française de Microbiologie
SRGA	Swedish Reference Group of Antibiotics

Amino Acid Abreviations:

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
SDN	Serine, Aspartic Acid, Asparagine
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
Xaa	Variable amino acid

β-Lactamase Abreviations:

BEL	Belgium ESBLs
BES	Brazilian ESBLs
BIL	Named After the Patient Bilal
CMS	Complex Mutant SHV
CMT	Complex Mutant TEM
CMY	Active on Cephamycins
CTX-M	Active on Cefotaxime, First Isolated at Munich
GES	Guyana ESBLs
GIM	German Imipenemase
IBC	Integron-Borne Cephalosporinase
IMP	Active on Imipenem
IRS	Inhibitor Resistant SHV
IRT	Inhibitor Resistant TEM
KLUA	From *Kluyvera ascorbata*
KLUC	From *Kluyvera cryocrescens*
KLUG	From *Kluyvera georgiana*
LAT	Named After Patient
LEN	From *K. pneumoniae* Strain LEN-1
MIR	Discovered at Miriam Hospital
OKP	Other *K. pneumoniae* β-Lactamase
OXA	Active on Oxacillin
PER	Pseudomonas Extended Resistant
PIT	From the Author's Name Pitton
PSE	Psedomonas-Specific Enzyme
SFO	*Serratia fonticola*
SHV	Sulphydryl Reagent Variable
TEM	Named After Patient Temoniera
TLA	Tlahuicas (Indian tribe)
VEB	Vietnam ESBLs
VIM	Verona Integron-Encoded Metallo-β-Lactamase

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CHAPTER I

General Introdution

1. Historical perspective

The initial discovery of antibiotics is generally attributed to Alexander Fleming in 1928, whose observations of a diffusible bacteriolytic substance produced by a mould strain permitted the identification of penicillin (Fleming, 1929). Using the filtrate of liquid cultures of *Penicillium notatum*, he determined the antibacterial activity of this antibiotic *in vitro*, as well as its non-toxicity when injected into mice and rabbits. Nevertheless, since no studies with bacterial infected animals were performed, Fleming failed to demonstrate the penicillin's ability to overcome these types of infections (Rolison, 1998).

With yet underdeveloped techniques, attempts to obtain purified penicillin in the 1930s were mostly unsuccessful, which would condemn the interest in this drug. However, a study in 1940 showed what Fleming failed to demonstrate. Penicillin was highly effective against a streptococcal infection in mice. The consequent introduction of penicillin into clinical use in humans motivated the discovery of other antibiotics. β-Lactams expansion began only in the early 1960s, with the development of the semisynthetic penicillins, followed by semisynthetic cephalosporins, as well as other β-lactam antibiotics (Rolison, 1998).

2. β-Lactams

2.1. Structure

β-Lactam antibiotics are composed of an isolated ring, as in monobactams, or associated in bicyclic ring structures in other classes. While penicillins are a group of natural or semisynthetic antibiotics, in which the β-lactam ring is fused with a thiazolidine ring, in cephalosporins the β-lactam ring are merged with a dihydrothiazine ring (Figure 1.1). In the carbapenems, the β-lactam ring is combined with a hydroxyethyl side chain, lacking an oxygen or sulphur atom in the bicyclic nucleus.

Overall, modifications of the R and R' groups (Figure 1.1), alter the pharmacokinetic and antibacterial properties in different β-lactam antibiotics. For example, modifications at position 7 of cephalosporins increase the penetration into the periplasmatic space and the stability against β-lactamases, but may reduce antibiotic activity (Donowitz & Mandell, 1988). In contrast to the antibiotic, the clavulanic acid, a β-lactamase inhibitor, is composed of a β-lactam ring fused with an oxazolidine ring and does not possessed an amide function.

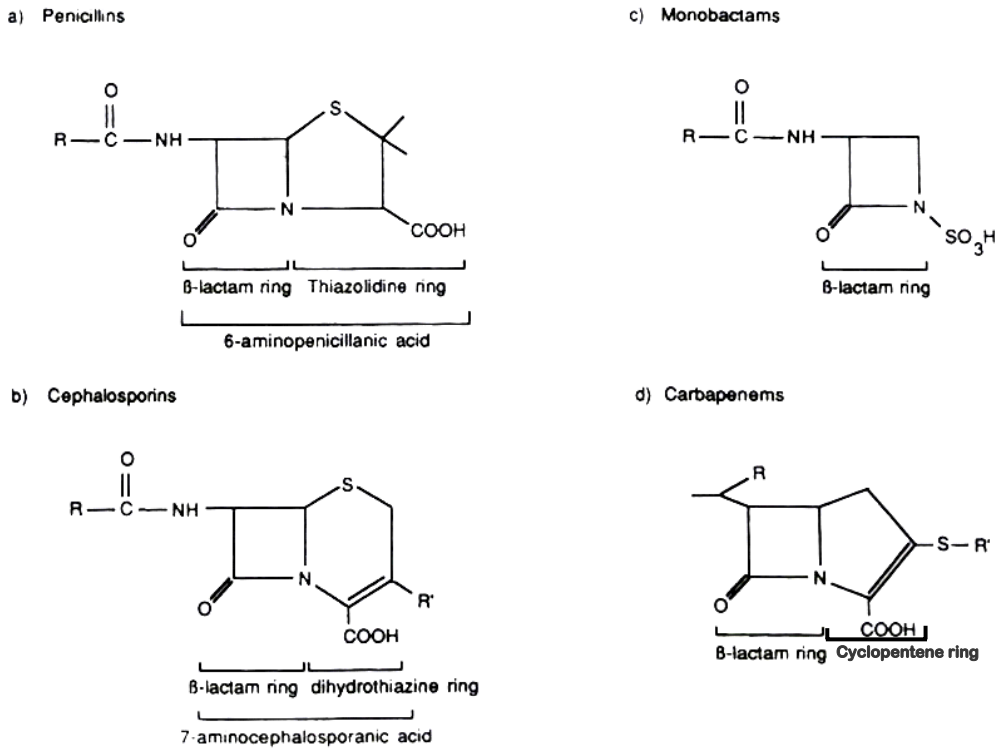

Figure 1.1 - Chemical structures of β-lactam antibiotics (adapet from Yao & Moellering, 2003).

2.2. Mechanism of action

β-Lactam antibiotics act on bacteria by inactivating the enzyme located in the citoplasmatic membrane which catalyses synthesis of the cross-linked peptidoglycan. Wall structure is different in Gram-positive and Gram-negative bacteria: in Gram-positive, a large layer of peptidoglycan involving the cell is followed by the citoplasmatic membrane, while in Gram-negative an outer membrane involves a thin layer of peptidoglycan followed by the inner or citoplasmatic membrane.

The peptidoglycan is composed of alternating chains of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) linked by β-(1,4)-glycoside units. The carboxyl group of muramic acid is usually replaced by an aminoacid chain composed of four aminoacids. The most common are L-alanine, D-alanine, D-glutamic acid, D-glutamine and L-lysine or diaminopimelic acid (DAP). Cross-linkage occurs directly in Gram-negative organisms between the diamino residues of one peptide chain and the D-alanine of the adjacent peptide chain. This cross-linking with the elimination of the terminal D-alanine is designated the transpeptidase reaction. D,D-carboxypeptidases are responsible for the removal of the terminal D-alanine from the peptide chain, which could become a donor in the transpeptidation reaction. These penicillin-binding proteins (PBPs), transpeptidase and

carboxypeptidase, are subject to inhibition by β-lactam antibiotics, because penicillins act as analog of D-alanyl-D-alanine (Tipper & Strominger, 1965). These transpeptidases and carboxypeptidases react with acyl-D-alanyl-D-alanine to form the acyl-D-alanyl-enzyme complex, with the elimination of the terminal D-alanine. β-Lactam-enzyme complex, formed after interaction between the enzyme and the β-lactam antibiotic, would act as competitor to the formation of the normal acylated enzyme. The β-lactam-enzyme complex is very stable, and culminates with the inactivation of the PBP's functions (Ghuysen, 1988). This interference with the normal cross-linking in the cell wall results in cellular lysis. Besides having transpeptidade and carboxypeptidase functions, PBPs also present a transglycosylase function, responsible for the polymerization of the glycan chain in peptidoglycan, which is not sensitive to β-lactams (Waxman & Strominger, 1983).

Different bacteria species present diverse PBPs, which are polypeptides with 40 to 120 kDa. Generally, in *Enterobacteriaceae*, the number of PBPs varies from six to eight, *Escherichia coli* specifically shows seven enzymes and *Klebsiella pneumoniae* presents six (Georgopapadakou & Lin, 1980; Spratt, 1983). In *E. coli*, the inactivation of PBPs with higher molecular weight, normally with transpeptidase or transglycosylase functions, ultimately leads to bacterial death. While inhibition of PBP1 leads to cellular lysis (Spratt, 1983), the inhibition of PBP2 results in the formation of spherical cells and that of PBP3 to the formation of filaments (Spencer *et al.*, 1987). The inactivation of PBPs with lower molecular weight, normally with carboxypeptidase functions, does not seems lethal to the cell (Spratt, 1983).

PBPs present different affinities towards β-lactams, according to the substitutions present in the β-lactam ring. Thus, in *E. coli*, penicillin derived β-lactams show higher affinities to PBP1 (amoxicillin), PBP2 (mecillinam and piperacillin), PBP3 (mezlocillin and piperacillin) and to PBP4, while all cephalosporins show higher affinities to PBP1, and some to PBP2 and PBP3 (cefotaxime) or just to PBP3 (ceftazidime) (Bryan & Godfrey, 1991).

Similarly to the β-lactamases from class A, C and D, PBPs possess a serine residue in the active site responsible for binding (Ambler, 1980; Jaurin *et al.*, 1981;Joris *et al.*, 1988; Ouellette *et al.*, 1987). This active site is composed of three conserved motifs responsible for the catalytic activity of these enzymes: Ser-Xaa-Xaa-Lys (which contains the active site serine and Xaa correspondes to a variable amino acid), (Ser/Tyr)-Xaa-Asn and (Lys/Arg/His)-(Thr/Ser)-Gly (Frère *et al.*, 1991). Forming the active site in the tertiary structure by folding of the polypeptide chain, these motifs are always disposed in the same order and with similar spacing in the primary protein structure (Ghuysen, 1991; Goffin & Ghuysen, 1998; Massova & Mobashery, 1998).

While in serine β-lactamases and lower molecular weight PBPs the active-site serine is situated close to the amino terminus of the peptide, in higher molecular weight PBPs the

active-site serine is located more centrally (Ghuysen, 1988; Keck *et al.*, 1985). In all of these types of enzymes, the conservation of motifs within the active-site and in other specific regions of the sequence may play an important role in the three-dimensional orientation of the active-site to form the presumptive active centre (Bryan & Godfrey, 1991).

2.3. Mechanisms of resistance to β-lactam antibiotics

Resistance to β-lactam antibiotics is still a continuing problem in therapeutic failure. Since the introduction of penicillin, this phenomenon aroused and continues to grow. With the introduction of penicillin into clinical use in humans, the first enzyme capable of destroy this antibiotic appeared (Abraham & Chain, 1988). With the introduction of semisynthetic penicillins in 1950-60, followed by cephalosporins as well other β-lactam antibiotics in 1970-80, bacteria continued to adapt and evolve, using mechanisms of mutations, genetic transference or natural selection (Frère *et al.*, 1991; Jacoby & Archer, 1991).

Therefore, there are four independent mechanisms of resistance to β-lactam antibiotics that can also act together. A first mechanism is target modifications, which implies a decrease in affinity, resulting in a low level of resistance. A second mechanism is based on the permeability of the outer membrane and implies a reduction of the penetration rates of the β-lactam, resulting in an intermediate level of resistance. A third mechanism is based on efflux pumps and implies the transport of the antibiotic from within the cell to the external environment, resulting in an intermediate level of resistance. A fourth mechanism, by far the most important and concerning mechanism of resistance, is the production of enzymes responsible for the hydrolysis of the antibiotic present in the periplasmatic space.

2.3.1. PBPs modifications

Modifications of PBPs have been described in both Gram-negative and Gram-positive organisms, assuming a more important role in Gram-positive bacteria. These modifications are associated with at least four mechanisms. A first mechanism is an aminoacid substitution that can reduce the affinity of the enzyme towards β-lactam. Nevertheless, the protein must remain capable of interacting with the D-alanyl-D-alanine under the risk of losing usefulness to the bacteria. However, since the majority of β-lactams have a large spectrum towards PBPs, aminoacid modifications in one or two enzymes will have very little effect on the resistance level. In *E. coli*, three different aminoacid substitutions within the transpeptidase domain of the PBP3, conferred resistance to cephalexin but not to other cephalosporins, penicillins or monobactams (Hedge & Spratt, 1985; Frère *et al.*, 1991).

A second mechanism is the acquisition of foreign PBP resistant to β-lactam antibiotics. Nevertheless, this acquired foreign PBP have the same capability of performing many of the funcions of a normal PBP. The emergence of methicillin-resistant *Staphylococcus aureus*, where an acquired PBP2a confers resistance to β-lactam antibiotics, is a good example (Hackbarth & Chambers, 1989).

A third mechanism of resistance is through recombination between susceptible PBPs and those of less susceptible species. This hybrid protein, is originated in an interspecies homologous recombination, presents a slightly less susceptibility to β-lactams (Dougherty, 1986).

Overexpression of a PBP is fourth mechanism. When PBP5 is overexpressed in *Enterococcus hirae*, increased levels of penicillin resistance are detected (Fontana *et al.*, 1983). In another study, strains with 50-fold increase of PBP3, in *E. coli*, showed increased of minimal inhibitory concentrations (MICs) of growth for aztreonam and ceftazidime (Hedge & Spratt, 1985)

2.3.2. Permeability-based resistance

Another mechanism of resistance is the decrease of permeability of the outer membrane. If in Gram-positive bacteria the β-lactam can easily reach the citoplasmatic membrane, in Gram-negative the crossing of the outer membrane is essentially done through protein channels, the porins (Nikaido, 1989). This outer membrane acts as a barrier to hydrophobic compounds in general and for hydrophilic compounds that exceed a low molecular weight.

β-Lactams penetration is done essentially by porins OmpF and OmpC, which are proteins inserted into the outer membranes that act as water-filled, non-specific, transmembrane diffusion channels for hydrophilic molecules (Sawai *et al.*, 1982). Being larger than OmpC, the OmpF channel is a major responsible for the penetrations of β-lactam antibiotics. Bacteria where the gene *ompF* is mutated, resulting in a modified OmpF, present a low level of resistance to β-lactam and the diffusion occurs through OmpC (Yoshimura & Nikaido, 1985).

Overall, hydrophobicity, size and charge of the molecule influence the rate of penetration. As an example, cephalosporins, which are less hydrophobic than penicillins, possess a better penetration rate. Nevertheless, modifications of the R and R' groups of each antibiotic may imply differences in the penetration rates (Nikaido, 1989).

2.3.3. Efflux pump

The third mechanism involved in resistance to β-lactam antibiotics is the expression of efflux pumps. These proteins transport the antibiotic from within the cell to the external environment (Webber & Piddock, 2003). A characteristic of efflux pumps is the variety of molecules they may transport, due to poor substrate specificity. Thus, this multidrug efflux system plays an important role in providing resistance to a very wide range of compounds in Gram-negative bacteria (Nikaido, 1996).

One of these multidrug efflux pumps is the resistance nodulation division (RND), a secondary active transporter. Examples of this superfamily include the AcrAB system of *E. coli* and the MexAB-OprM system of *Pseudomonas aeruginosa* (Li *et al.*, 1995; Ma *et al.*, 1993). Previous studies pointed out that the inactivation of the specific components of efflux pumps decrease the MIC values of some β-lactams. For example, inactivation of *mexA*, in *P. aeruginosa*, decreased the MICs of ceftriaxone, cefoperazone, azlocillin and carbenicillin (Li *et al.*, 1995). Another study suggests that clavulanic acid was also accommodated by the MexAB-OprM system (Li *et al.*, 1998). In *E. coli*, deletion of *acrAB* decreased MIC values of ampicillin and benzylpenicillin (Ma *et al.*, 1993). Overall, these results appear to suggest that such systems increase the resistance to β-lactam antibiotics.

2.3.4. Enzyme production

The fourth and most important mechanism of resistance to β-lactam antibiotics is the production of enzymes capable of structurally modifying the antibiotic. These enzymes constitute three major groups: the esterases, which hydrolyse the C3 acetil group of the dihydrothiazine ring in cephalosporins, originating a product with reduced antibiotic activity; the acilases, which hydrolyse the amide bond of the side-chain of penicillins and cephalosporins and which are more important *in vitro*, for the production of semi-synthetics penicillins, than *in vivo* (Sykes & Matthew, 1976); the third and by far the most important group of this type of enzymes is the β-lactamases, which irreversibly inactivate the antibiotic, hydrolysing the cyclic amide bond of the β-lactam ring. With penicillins, the product of the reaction is the penicilloic acid, while with cephalosporins it is cephalosporoic acid. Reactions catalysed by β-lactamases are the most important mechanism of resistance to β-lactam (Livermore, 1995).

3. β-Lactamases

Originally designated by the Nomenclature Committee of the International Union of Biochemistry as "enzymes hydrolysing amides, amidines and other C-N bonds … separated on the basis of the substrate: … cyclic amides", the β-lactamases (EC 3.5.2.6) are the most common cause of bacterial resistance to β-lactam antimicrobial agents (Webb, 1984). With the discovery of the first β-lactamase in 1940, prior to the release of penicillin for clinical practice, it became rapidly apparent that bacteria were already one step ahead (Abraham & Chain, 1988). In 1965, a plasmid-mediated β-lactamase isolated from a patient named Temoniera presented ampicillin resistance and was subsequently designated TEM (Datta & Kontomichalou, 1965).

3.1. Classification

Several classification schemes for β-lactamases have been developed over the years. A first consensual classification scheme was proposed in 1968, with penicillinases and cephalosporinases (Sawai *et al.*, 1968). In 1970, Jack & Richmond (1970) proposed another classification scheme, which was updated in 1973 by Richmond & Sykes (1973), with five major groups based on the substrate profile. In 1976, Sykes & Matthew (1976) extended this last scheme to include plasmid-mediated β-lactamases differentiated by isoelectric focusing (IEF). In 1981, Mitsuhashi and Inoue (1981) proposed a scheme where they added the category "cefuroxime-hydrolyzing β-lactamase" to the "penicillinase and cephalosporinase" classification. However, with the discovery of new enzymes every year, it became necessary to propose a new classification. In 1989, Bush (1989a; 1989b; 1989c) updated the existing classification and tried to correlate substrate and inhibitory properties with molecular structure. Molecular structure classification was first proposed by Ambler in 1980 with classes A and B (Ambler, 1980). In 1981, Jaurin & Grundstrom described class C (Jaurin & Grunström, 1981), and in the late 1980s, class D, constituting oxacillin-hydrolyzing enzymes, were segregated from the other serine β-lactamases (Huovinen *et al.*, 1988; Ouellette *et al.*, 1987). Finally, in 1995, new entries were done to the Bush classification scheme of 1989 (Bush *et al.*, 1995). This last scheme, updated in 1995, continues to attempt to correlate the functional characteristics with the molecular structure (Table 1.1). The four groups of β-lactamases are:

- Group 1: Chromosome encoded cephalosporinases that are not fully inhibited by clavulanic acid;

- Group 2: β-Lactamases belonging to classes A and D that are generally inhibited by active site-directed β-lactamase inhibitors;
- Group 3: Metallo-β-lactamases that hydrolyse penicillins, cephalosporins and carbapenems, not fully inhibited by β-lactamase inhibitors, except for ethylenediaminetetraacetic acid (EDTA) and *p*-chloromercuribenzoate (pCMB), which require the presence of zinc or another heavy metal;
- Group 4: Penicillinases that are not inhibited by clavulanic acid.

When compared with the classification proposed by Bush in 1989, this new proposal suggests the formation of two sub-groups derived from 2b: the 2be sub-group, which includes a growing number of enzymes from the TEM, SHV and, more recently, the CTX-M family; and the 2br sub-group, which includes enzymes with reduced affinity to β-lactamase inhibitors. The new proposal also suggests the formation of group 2f, consisting of carbapenems-hydrolysing enzymes closely related to metallo-β-lactamases from group 3, but with an active site serine.

This last classification scheme is widely accepted, as is Ambler's proposal for molecular structure. As such, this work also follows these classification schemes (Table 1.1).

3.2. Active site

The four classes defined by the molecular structure, A to D, have essential differences in their active sites, responsible for their different catalytic properties. While in classes A, C and D an active site serine is responsible for the β-lactam hydrolysis, in class B, enzymes require zinc or another heavy metal to catalyse antibiotics.

3.2.1. Serine β-lactamases

Class A enzymes display four conserved motifs that create a complex hydrogen-bond network to fix the β-lactam in the substrate-binding pocket or active centre (Matagne *et al.*, 1998). Residues Ser70-Xaa-Xaa-Lys73, Ser130-Asp131-Asn132 (SDN Loop), and Lys/Arg234-Thr/Ser235-Gly236 define the conserved residues which are essential for β-lactam binding and hydrolysis.

Table 1.1 - Updated classification scheme for bacterial β-lactamases (adapted from Bush *et al.*, 1995)

Bush-Jacoby-Medeiros group	Molecular class[a]	Preferred substrates	Inhibyted by		Representative enzymes
			CA[b]	EDTA	
1	C	Cephalosporins	-	-	AmpC enzymes from Gram-negative bacteria; MIR-1
2a	A	Penicillins	+	-	Penicillinases from Gram-positive bacteria
2b	A	Penicillins, cephalosporins	+	-	Broad-spectrum: TEM-1, TEM-2, SHV-1
2be	A	Penicillins, narrow-spectrum and extended-spectrum cephalosporins, monobactams	+	-	Extended-spectrum: TEM-3 to TEM-160, SHV-2 to SHV-101, *K. oxytoca* K1, CTX-M family
2br	A	Penicillins	±	-	Inhibitor resistant TEM (IRT) TEM-30 to TEM-36, Inhibitor resistant SHV (IRS) SHV-10, SHV-26, SHV-49
2c	A	Penicillins, carbenicillin	±	-	PSE-1, PSE-3, PSE-4
2d	D	Penicillins, cloxacillin	±	-	OXA-1 to OXA-11, PSE-2 (OXA-10)
2e	A	Cephalosporins	+	-	Inducible cephalosporinases from *Proteus vulgaris*
2f	A	Penicillins, cephalosporins, carbapenems	+	-	NMC-A from *Enterobacter cloacae*, Sme-1 from *Serratia marcescens*
3a	B (Metalloenzymes)	Most β-lactam, including carbapenems	-	+	Zinc-dependent carbapenemases: IMP, VIM
3b	B				L1 from *Xanthomonas maltophilia*, CcrA from *Bacteroides fragilis*
3c	B				
4	ND[c]	Penicillins	-	?	Penicillinase from *Pseudomonas cepacia*

[a] Date from Ambler, 1980; Jaurin & Grundström, 1981; Huovinen *et al.*, 1988.
[b] CA, clavulanic acid.
[c] ND, not determined.

In the active centre, the oxyanion hole, defined by the NH group backbone of Ser70 and Ala237, is also used to attract β-lactams for hydrolysis (Knox, 1995) (Figure 1.2). Amino acids 160 to 179 define the Ω loop, where Glu166 acts as a general base in the catalytic process (Strynadka *et al.*, 1992).

Compared to class A, class C enzymes possess a larger active centre, which allows the binding of the bulky extended-spectrum cephalosporins (oxyimino-β-lactams) (Crichlow *et al.*, 1999). This conformational flexibility facilitates the hydrolysis of oxyimino-β-lactams by providing more space for water molecules to attack the acyl-enzyme intermediate. As in class A β-lactamases, the first element contains the active site serine (Ser-Xaa-Xaa-Lys), a second element contains a Ser-Xaa-Asn or Tyr-Xaa-Asn pattern that corresponds to the SDN loop of class A enzymes and on the opposite side of the active-site, a third element contains (Lys/Arg/His)-(Thr/Ser)-Gly. The Ω loop of class C enzymes is not readily apparent, but its function could be performed by the tyrosine of the second element.

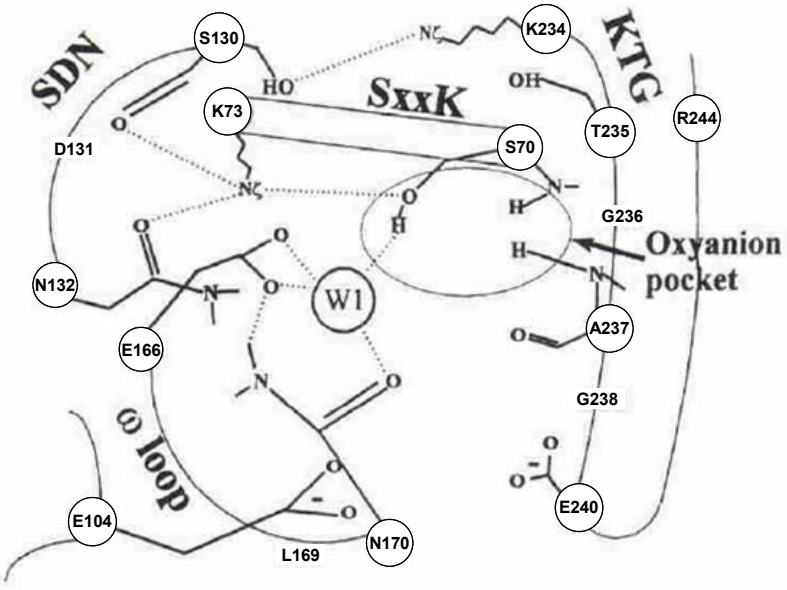

Figure 1.2 - Schematic diagram showing the hidrogen bonding site motifs of a class A β-lactamase (adapted from Medeiros, 2000).

Segregated from the other class with active site serine in the late 1980s, class D enzymes possess only 16% of aminoacid identities with class A or class C enzymes. Nevertheless, a significant characteristic is the practically perfect symmetry between class A and class D enzymes for all atoms that constitute the catalytic mechanism for acylation

(example: Ser67, Lys70, Ser115 and Lys205 in OXA-10 and Ser70, Lys73, Ser130, and Lys234 in TEM-1, respectively). However, an extension of the binding site seems to exist in OXA-10, were the “oxyanion hole” is provided by the main chain nitrogen atoms of Ser67 and Phe208. Another difference from class A enzymes is the involving of the Lys70 residue in acylation and deacylation (Maveyraud *et al.*, 2000).

3.2.2. Metallo-β-lactamases

Class B metallo-β-lactamases require a zinc or another type of heavy metal ion to hydrolyse β-lactam antibiotics, and their activity is inhibited by chelating agents like EDTA or pCMB (Rasmussen & Bush, 1997). At least two models have been proposed for chromosomal metallo-β-lactamases from *Bacillus cereus* and from *Bacteroides fragilis*. The model proposed for metallo-β-lactamase II from *B. cereus* presents a structure where the histidine residues at positions 86, 88 and 149, act as ligands to a single Zn^{2+} moiety located within the active site (Carfi *et al.*, 1995) (Figure 1.3). First, this metal ion activates the water molecule for nucleophilic attack on the peptide carbonyl, in which Asp90 acts as a general base to remove a proton from the water molecule and, secondly, the Zn^{2+} binds and polarizes this carbonyl group. Asp90 and Cys168 maintain the position of the water molecule which allows the nucleophilic attack.

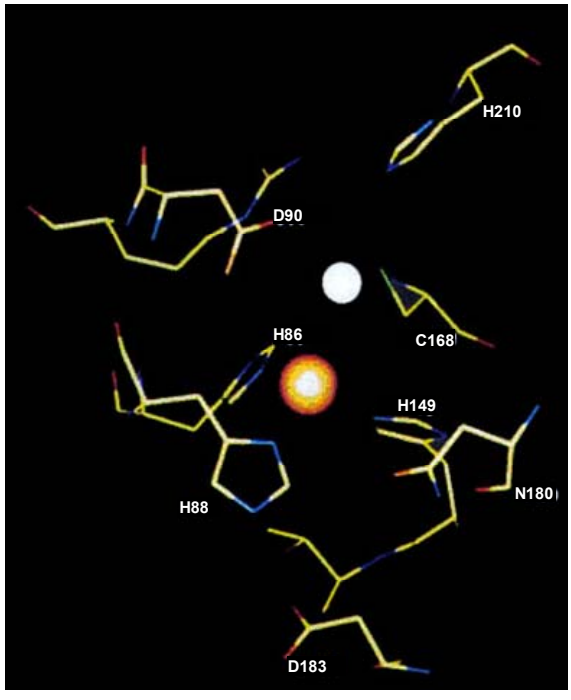

Figure 1.3 – Active site of the metallo-β-lactamase present in *B. cereus*. The Zn^{2+} ion and the water molecule (Wat1) are represented by an orange and a white sphere, respectively (Adapted from Carfi *et al.*, 1995).

The model proposed for metallo-β-lactamases from *B. fragilis* is based on the presence, at the active site, of two zinc atoms sharing a water (hydroxide) moiety that serves as a ligand for both ions (Concha *et al.*, 1996). It was proposed that this hydroxide could be responsible for the nucleophilic attack on the carbonyl carbon of the β-lactam. The tetrahedral intermediate which is formed is thought to be stable as a result of the formation of an oxyanion hole, flanked by the side chain of the conserved Asn193 and the tetrahedral zinc (Concha *et al.*, 1996).

3.3. Structure, properties, conferred resistance and distribution of different β-lactamase families

β-Lactamases can be found encoded in the chromosome, plasmids or transposons and produced in a constitutive or inducible mode. With the exception of the *Salmonella* genus, chromosomal β-lactamases are almost always ubiquitous in *Enterobacteriaceae* (Livermore, 1995). Whether constitutive or inducible, the production of chromosomal β-lactamases varies in quantity between low and high production levels, thus affecting the degree of resistance conferred (Table 1.2).

Clinical relevant enzymes confer resistance to a wide range of β-lactams and combination of β-lactam with β-lactamase inhibitors. Several types of chromosomal enzymes are expressed by *Enterobacteriaceae*. However, three major groups are of special importance: AmpC β-lactamases, which are constitutive in *E. coli* and *Shigella*; SHV, LEN and OKP enzymes, constitutive in *K. pneumoniae*; and KLUA, KLUC and KLUG β-lactamases, which are constitutive in *Kluyvera ascorbata*, *K. cryocrescens* and *K. georgiana*, respectively, and probably the progenitors of some plasmid-mediated CTX-M enzymes (Decousser *et al.*, 2001; Humeniuk *et al.*, 2002, Poirel *et al.*, 2002c).

Resistance to β-lactams increased with the natural selection of bacteria expressing β-lactamases after the general introduction of antibiotics in clinical practice. This increase was also due to the spread of self-transferable plasmids responsible for resistance phenotypes. The report of the first TEM-type enzyme conferring transferable ampicillin resistance was followed, in 1974, by the identification of a transposon containing a bla_{TEM} gene as the first mobile element responsible for dissemination of ampicillin resistance (Datta & Kontomichalou, 1965; Hedges & Jacob, 1974). Almost 20 years later, in 1992, the first transposon carrying genes that encode extended-spectrum β-lactamases (ESBL), which confer resistance to third generation cephalosporins, was described (Heritage *et al.*, 1992). Confirming this idea is the association between bla_{CTX-M} genes, which code for one of the most important families of β-lactamases, and insertion sequences located upstream and

downstream in the majority of *bla* genes of this family (Bonnet, 2004). This overall very recent dissemination of plasmid-mediated β-lactamases in Gram-negative bacteria is a good example of fast-paced evolution.

Table 1.2 - Chromosomal β-lactamases and their expression in enterobacteria (adapted from Livermore, 1995)

Organism	Name	Class	Bush group	Mode of expression[a]			
				Inducible	Constitutive		
					Minimal	Moderate	High
E. coli	AmpC	C	1	-	●	-	○
Shigellae	AmpC	C	1	-	●	-	○
Enterobacter spp.	AmpC	C	1	●	○	-	●
Citrobacter freundii	AmpC	C	1	●	○	-	●
Morganella morganii	AmpC	C	1	●	-	-	●
Providencia spp.	AmpC	C	1	●	○	-	○
Serratia spp	AmpC	C	1	●	-	-	●
K. pneumoniae	SHV-1	A	2b	-	-	●	○
Klebsiella oxytoca	K1	A	2be	-	-	●	●
Citrobacter diversus		A[b]	2e	●	○	-	○
Proteus vulgaris	Cxase[c]	A	2e	●	-	-	○
Proteus penneri	CXase	A	2e	●	-	-	○
Proteus mirabillis		?[d]	?[d]	-	●	-	-

[a] ●, Normal mode of production, typical of the species; ●, frequently encountered, variable among countries, hospitals, and units, but seen in 10 to 50% of isolates in most recent surveys; ○, rare, seen in fewer than 10% of isolates; -, unknown or isolated reports only. Minimal production: denotes that enzyme is detectable but causes no significant resistance; moderate: denotes that the enzyme contributes to resistance to good substrates; high: denotes huge levels of enzyme (up to 3% of total cell protein in some *Enterobacter*) able to confer resistance even to weak substrates.
[b] Production of class C enzymes has been seen in a few *C. diversus* isolates (Jones *et al.*, 1994).
[c] CXase, cefuroximase belonging to Bush-Jacoby-Medeiros's group 2e (Bush *et al.*, 1995).
[d] No particular chromosomal β-lactamase has been defined as being typical of *P. mirabilis*.

3.3.1. AmpC β-lactamases

AmpC β-lactamases are chromosome-encoded cephalosporinases found in *E. coli*, which are not strongly inhibited by clavulanic acid, and that can be found in group 1 of Bush-Jacoby-Medeiros classification scheme and in class C according to molecular structure (Bush *et al.*, 1995; Jaurin & Grundström, 1981). With minimal expression in *E. coli* (Normak *et al.*, 1980), these enzymes do not confer resistance to ampicillin and first generation cephalosporins, thus being rarely involved in clinical failure of β-lactams. This is because the gene *ampC*, which encodes for AmpC β-lactamase, is regulated by a weak promotor (Olsson *et al.*, 1983) and a transcription attenuator (Jaurin *et al.*, 1981). In *E. coli*, the *ampC* gene overlaps the fumarate reductase (*frdD*) gene, with the transcriptional terminator for the *frdD* operon located in the *ampC* gene, acting as an attenuator for this β-lactamase encoding

gene (Jaurin *et al.*, 1981). When *ampC* is preceded by a strong promotor, an increase in the transcription fosters the hyperproduction of AmpC β-lactamases (Nelson & Elisha, 1999). Two separate mutations in *E. coli* also induce the hyperproduction of this type of enzymes (Bennett & Chopra, 1993). Overall, hyperproducers of AmpC enzymes account to less than 2% of *E. coli* strains in several studies (Livermore, 1995).

Detection of plasmid-mediated *ampC* genes in *Enterobacteriaceae* that produce AmpC-type β-lactamases has become quite common with the identification of MIR-1 (discovered at Miriam Hospital) and CMY-1 (active on cephamycins) in *K. pneumoniae*, BIL-1 (named after the patient Bilal), LAT-3 and LAT-4 (named after patient) in *E. coli*, among others (Bauernfeind *et al.*, 1989; Fosberry *et al.*, 1994; Gazouli *et al.*, 1998; Papanicolaou *et al.*, 1990). In general, plasmid-mediated AmpC enzymes fall into at least six phylogenetic groups (Perez-Perez & Hanson, 2002).

The occurrence of plasmid-mediated AmpC β-lactamases has been reported worldwide (Coudron *et al.*, 2000; Li *et al.*, 2008; Muratani *et al.*, 2006; Song *et al.*, 2006; Woodford *et al.*, 2007). The most successful plasmid-mediated AmpC β-lactamases – the CMY enzymes – are derived from the chromosomal cephalosporinases of *Citrobacter freundii* (Miriagou *et al.*, 2004). The association of CMY-encoding genes with mobile elements, such as *orf513* or IS*26*, may be the reason behind this worldwide spread (Chen *et al.*, 2007).

Overall, strains producing plasmid-mediated AmpC enzymes are generally resistant to penicillins, extended-spectrum cephalosporins, monobactam and cephamycins, but are susceptible to cefepime, cefpirome and carbapenems (Philippon & Jacoby, 2002). Nevertheless, it is very difficult to distinguish between plasmid-mediated AmpC-producing organisms and ESBL-producing organisms using phenotypic susceptibility testing methods alone.

3.3.2. TEM β-lactamases

The TEM family is the most diverse family of β-lactamases, presenting over than 163 different enzymes, according to the internet site of the Lahey Clinic (http://lahey.org/studies/temtable.asp), maintained by Prof. George Jacoby and Prof. Karen Bush and last updated in 20 of August 2008.

The first member belonging to the TEM family, TEM-1, is able to hydrolyse ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, presents negligible activity against extended-spectrum cephalosporins, and is inhibited by clavulanic acid. Considered as one of the parental or progenitor enzymes of ESBL-TEM enzymes, plasmid-mediated TEM-1 is by far the most prevalent β-lactam inactivating enzyme found in enteric bacilli (Liu

et al., 1992). The second parental enzyme of this family is TEM-2, which differs from TEM-1 in an aminoacid substitution at position 39 (Gln39Lys), but confers a similar phenotype of resistance (Ambler & Scott, 1979; Jacoby & Sutton, 1985; Jacoby & Carreras, 1990).This substitution changes the isoelectric point from 5.4 in TEM-1 to 5.6 in TEM-2.

Another difference between TEM-1 and TEM-2 is the strength of the promoters upstream of the bla_{TEM} genes, which encode for these β-lactamases. The bla_{TEM-2} gene presents a promoter which is stronger than the promoter of the bla_{TEM-1} gene. Bacterial strains expressing a TEM-2 enzyme present a higher enzymatic activity when compared to TEM-1 producing strains (Chaïbi *et al.*, 1996; Jacoby & Carreras, 1990). Another feature related to the genetic environment of the bla_{TEM} genes is found in the bla_{TEM-6} gene, which presents a 116 bp IS*1*-like element upstream of the coding region (Goussard *et al.*, 1991). This element is responsible for the high-level synthesis of this enzyme. TEM variants are derived from parental enzymes TEM-1a to TEM-1h and TEM-2, and from these TEM-1a, TEM-1b and TEM-2 are encoded in three transposons, Tn*3*, Tn*2* and Tn*1*, respectively (Archambault *et al.*, 2006; Partridge & Hall, 2005).

The majority of the TEM family is composed of ESBL enzymes which are derived from parental enzymes TEM-1 and TEM-2 (Table 1.3). Specific amino acid substitutions at positions Asp104, Arg164, Ala237, Gly238 and Glu240 cause variations in the level of resistance to several compounds and confer the ESBL phenotype. As an example, TEM-3 with substitutions Gln39Lys, Asp104Lys and Gly238Ser provides resistance to all extended-spectrum cephalosporins and monobactams (Jacoby & Carreras, 1990). In SHV enzymes, the presence of the Gly238Ser in the active site pushes the β-strand out and away from the reactive Ser70 residue, resulting in a slightly expanded active site, which could improve binding and accommodate cephalosporins with bulky side-chains (Huletsky *et al.*, 1993; Matagne *et al.*, 1998).

On the other hand, TEM-10, which presents Arg164Ser and Glu240Lys substitutions, hydrolyses ceftazidime preferably over cefotaxime (Quinn *et al.*, 1989). This last mutation is responsible for the increase in hydrolysing activity against ceftazidime, based on a change of the electrostatic charge of the exposed group at position 240 (Huletsky *et al.*, 1993; Randegger *et al.*, 2000).

Although mutation Glu104Lys improves the ability of the enzyme to hydrolyse cephalosporins and monobactams, it is not sufficient to confer true resistance. When associated with at least one mutation in another part of the active site (e.g., Arg164Ser), the combined effect of the two mutations is responsible for increasing the hydrolytic capability of the enzyme towards third-generation cephalosporins (Sowek *et al.*, 1991).

Table 1.3 - TEM enzymes presenting amino acid substitutions associated with ESBL phenotype (according to website http://lahey.org/studies/temtable.asp)

β-Lactamase	Amino Acid at position																							pI[a]
	6	21	35	39	42	49	51	92	104	153	163	164	173	175	179	182	196	224	237	238	240	265	268	280	
TEM-1	Gln	Leu	Asp	Gln	Ala	Leu	Leu	Gly	Glu	His	Asp	Arg	Ile	Asn	Asp	Met	Gly	Ala	Ala	Gly	Glu	Thr	Ser	Ala	5.4
TEM-2				Lys																					5.6
TEM-3				Lys					Lys											Ser					6.3
TEM-4		Phe							Lys											Ser		Met			5.9
TEM-5												Ser							Thr		Lys				5.55
TEM-6									Lys			His													5.9
TEM-7				Lys								Ser													5.4
TEM-8				Lys					Lys			Ser								Ser					5.9
TEM-9		Phe							Lys			Ser										Met			5.5
TEM-10												Ser									Lys				5.6
TEM-11				Lys								His													5.6
TEM-12												Ser													nd
TEM-15									Lys											Ser					6.0
TEM-16				Lys					Lys			His													6.3
TEM-17									Lys																5.5
TEM-18				Lys					Lys																6.3
TEM-19																				Ser					5.4
TEM-20																Thr				Ser					5.4
TEM-21				Lys					Lys	Arg										Ser					6.4
TEM-22				Lys					Lys										Gly	Ser					6.3
TEM-24				Lys					Lys			Ser							Thr		Lys				6.5
TEM-25		Phe																		Ser		Met			5.3
TEM-26									Lys			Ser													5.6
TEM-27												His									Lys	Met			5.9
TEM-28												His									Lys				6.1
TEM-29												His													5.42
TEM-42				Lys	Val															Ser	Lys	Met			5.8
TEM-43									Lys			His				Thr									6.1

Continued

Table 1.3 - Continued

β-Lactamase	Amino Acid at position																							pI[a]
	6	21	35	39	42	49	51	92	104	153	163	164	173	175	179	182	196	224	237	238	240	265	268	280	
TEM-1	Gln	Leu	Asp	Gln	Ala	Leu	Leu	Gly	Glu	His	Asp	Arg	Ile	Asn	Asp	Met	Gly	Ala	Ala	Gly	Glu	Thr	Ser	Ala	5.4
TEM-46				Lys					Lys			Ser									Lys				6.5
TEM-47																				Ser	Lys	Met			6.0
TEM-48		Phe																		Ser	Lys	Met			6.0
TEM-49		Phe																		Ser	Lys	Met	Gly		6.0
TEM-52									Lys							Thr				Ser					6.0
TEM-53		Phe										Ser													nd
TEM-56				Lys					Lys	Arg															6.4
TEM-60				Lys			Pro		Lys			Ser													6.4
TEM-61				Lys								His									Lys				6.5
TEM-63		Phe							Lys			Ser				Thr									5.6
TEM-66				Lys				Asp	Lys											Ser					6.0
TEM-71																				Ser	Lys				6.0
TEM-72				Lys												Thr				Ser	Lys				5.9
TEM-75		Phe										His										Met			nd
TEM-85		Phe										Ser									Lys	Met			5.5
TEM-86		Phe										Ser							Thr		Lys	Met			5.5
TEM-87	Lys								Lys		His	Cys				Thr									nd
TEM-88									Lys							Thr	Asp			Ser					5.6
TEM-91												Cys				Thr					Lys				nd
TEM-92	Lys								Lys							Thr				Ser					6.0
TEM-93																Thr				Ser	Lys				6.0
TEM-94		Phe							Lys							Thr				Ser		Met			6.0
TEM-101				Lys																Ser	Lys			Val	nd
TEM-102		Phe										Ser										Met			nd
TEM-106									Lys							Thr									6.0
TEM-107									Lys			His				Thr				Ser					6.0

Continued

Table 1.3 - Continued

β-Lactamase	Amino Acid at position																							pI[a]
	6	21	35	39	42	49	51	92	104	153	163	164	173	175	179	182	196	224	237	238	240	265	268	280	
TEM-1	Gln	Leu	Asp	Gln	Ala	Leu	Leu	Gly	Glu	His	Asp	Arg	Ile	Asn	Asp	Met	Gly	Ala	Ala	Gly	Glu	Thr	Ser	Ala	5.4
TEM-111									Lys											Asp		Met			nd
TEM-112										Arg										Ser					5.4
TEM-113				Lys					Lys							Thr				Ser					6.3
TEM-114				Lys								Ser							Thr		Lys				5.9
TEM-115		Phe										His													nd
TEM-118												His										Met			nd
TEM-120		Phe																		Ser					nd
TEM-126															Glu	Thr									5.4
TEM-129				Lys					Lys			Ser													nd
TEM-130			Pro	Lys					Lys			Ser							Thr						nd
TEM-131		Phe							Lys			Ser				Thr			Thr						nd
TEM-132												His	Val								Lys				nd
TEM-133		Phe							Lys			Ser													nd
TEM-134				Lys					Lys			His								Ser					nd
TEM-136												Ser							Thr		Lys		Gly		nd
TEM-137												Ser									Arg				nd
TEM-138									Lys					Ile						Ser					nd
TEM-139				Lys		Met			Lys											Ser					6.3
TEM-142									Lys											Asn		Met			nd
TEM-143												Cys													nd
TEM-144												Cys									Lys				5.6
TEM-147												His						Val							nd
TEM-149									Lys			Ser				Thr					Val				nd

[a] Isoeletric point.
nd, not determined.

It has been suggested that the ammonium group of the long Lys side-chain interacts with the carboxylic group of the oximino substituents of ceftazidime and aztreonam (Knox, 1995). Another study suggests that Lys104 might modify the precise positioning of the SDN loop, which is involved in the binding and catalysis of the substrate (Petit *et al.*, 1995).

Amino acid substitutions at position Arg164 are the most common mutations in TEM enzymes (Table 1.3). Substitutions with either serine or histidine allow a greater flexibility of the Ω loop, opening the active site for bulky β-lactam variants. A TEM-1 mutant prepared by site-directed mutagenesis and containing the Arg164Ser substitution, showed increased catalytic efficiency for cefotaxime, ceftazidime and aztreonam (Sowek *et al.*, 1991).

Another amino acid substitution associated with the ESBL phenotype is the Ala237Thr. This substitution is found in TEM-5 and TEM-24 (Chanal *et al.*, 1992), and it improves binding of the enzyme to cefotaxime by facilitating the creation of a hydrogen bond between the Thr237 and the cefotaxime molecule (Kuzin *et al.*, 1995).

Besides potentially conferring an ESBL phenotype, TEM enzymes can also present reduced affinity to β-lactamase inhibitors, conferring an inhibitor resistant TEM (IRT) phenotype. These types of enzymes normally confer a phenotype of resistance to β-lactam/β-lactamase inhibitor combinations and susceptibility to third generation cephalosporins. On the other hand, IRT enzymes confer less resistance to narrow-spectrum cephalosporins than parental enzymes. Amino acid substitutions in TEM at positions Met69, Ser130, Arg244, Arg275 and Asn276 are usually associated with the resistance to β-lactamase inhibitors (Table 1.4).

The side chain of the Met69 residue forms the back hole of the oxyanion pocket, and the substitution of this residue by others of different size and degree of hydrophobicity could deform the attractive properties of the oxyanion hole (Knox, 1995). Furthermore, the Met69Ile substitution may move the amide side chain of Asn170 and Glu166, displacing the water molecule coordinated by Asn170, Ser70 and Glu166, thus diminishing the catalytic efficiency (Farzaneh *et al.*, 1996). Another study suggests that substitutions at Met69 could be responsible for the displacement of Ser130 or even Ser70 (Wang *et al.*, 2002).

Situated in the SDN loop, the Ser130 residue plays a crucial role in the structure and function of class A β-lactamases (Jacob *et al.*, 1990), particularly in the inactivation process by β-lactamase inhibitors (Bermudes *et al.*, 1999). By PCR mutagenesis of TEM-1, Ser130Gly substitution was showed to confer resistance to β-lactamase inhibitors, associated with a high decrease of ampicillin resistance (Vakulenko *et al.*, 1998).

Table 1.4 - TEM enzymes presenting amino acid substitutions associated with IRT phenotype (according to website http://lahey.org/studies/temtable.asp)

β-Lactamase (Secondary names)	Amino Acid at position																pI[a]
	21	39	69	80	114	127	130	165	182	196	221	244	262	265	275	276	
TEM-1	Leu	Gln	Met	Val	Thr	Ile	Ser	Trp	Met	Gly	Leu	Arg	Val	Thr	Arg	Asn	5.4
TEM-30 (IRT-2)												Ser					5.2
TEM-31 (IRT-1)												Cys					5.2
TEM-32 (IRT-3)			Ile						Thr								5.4
TEM-33 (IRT-5)			Leu														5.4
TEM-34 (IRT-6)			Val														5.4
TEM-35 (IRT-4)			Leu													Asp	5.2
TEM-36 (IRT-7)			Val													Asp	5.2
TEM-37 (IRT-8)			Ile													Asp	5.2
TEM-38 (IRT-9)			Val												Leu		5.2
TEM-39 (IRT-10)			Leu					Arg								Asp	5.4
TEM-40 (IRT-11)			Ile														5.4
TEM-44 (IRT-13)		Lys										Ser					5.4
TEM-45 (IRT-14)			Leu												Gln		5.2
TEM-51 (IRT-15)												His					5.2
TEM-54												Leu					nd
TEM-58												Ser	Ile				5.2
TEM-59 (IRT-17)		Lys					Gly										5.6
TEM-65 (IRT-16)		Lys										Cys					5.4
TEM-67	Ile	Lys										Cys					5.2
TEM-73 (IRT-18)	Phe											Cys		Met			5.2
TEM-74 (IRT-19)	Phe											Ser		Met			5.2
TEM-76 (IRT-20)							Gly										nd
TEM-77 (IRT-21)			Leu									Ser					nd
TEM-78 (IRT-22)			Val					Arg								Asp	nd
TEM-79												Gly					nd
TEM-80 (IRT-24)			Leu			Val										Asp	5.2
TEM-81			Leu			Val											nd
TEM-82			Val												Gln		nd
TEM-83			Leu					Cys							Gln		nd
TEM-84																Asp	nd
TEM-97			Val														nd
TEM-99												Ser					nd
TEM-103 (IRT-28)															Leu		5.2
TEM-108				Glu						Ser						Ser	nd
TEM-122															Gln		5.25
TEM-145											Met	His					nd
TEM-146					Pro							His					nd
TEM-159	Phe		Ile						Thr								nd
TEM-160		Lys	Val														nd

[a] Isoeletric point.
nd, not determined.

Another residue associated with the resistance to β-lactamase inhibitors, especially to clavulanic acid, is Arg244. In studies performed in inactivation by clavulanic acid, this conserved residue appears to be in position to induce a water molecule to provide proton for the formation of an acyclic intermediate (Knox, 1995). Substitutions of Arg244 by Cys or Ser

by site-directed mutagenesis were reported to confer resistance to inactivation by clavulanic acid (Imtiaz *et al.*, 1994). Clinical isolates of *E. coli*, resistant to amoxicillin plus clavulanic acid, were also reported (Vedel *et al.*, 1992). However, the association of double mutations Ser244 and Ser164 reverses the resistance to clavulanic acid (Imtiaz *et al.*, 1994).

Residue Arg275 is also believed to increase resistance to β-lactamase inhibitors when associated with Met69Leu (Caniça *et al.*, 1997a). In IRT-14 (Table 1.4), Arg275Gln is expected to destabilize the active site, due to a decrease of restriction in movements of Arg244, important in maintaining the integrity of the active site (Caniça *et al.*, 1997a). This mutation can be found in different enzymes other than IRT-14, such as TEM-82, TEM-83 and TEM-122. In this last enzyme, identified in the United States, the Arg275Gln substituition was found alone and confers an IRT phenotype (Kaye *et al.*, 2004).

Next to Arg275, the residue Asn276 has a direct effect on substrate affinities. The Asn276Asp amino acid substitution leads to a loss of affinity to clavulanic acid and to a slightly decrease of catalytic efficiencies (Saves *et al.*, 1995). With penicillins, catalytic efficiencies for the mutant enzyme carrying Asn276Asp were reduced two to six fold compared with TEM-1, while with cephalosporins, the mutant enzyme presented higher catalytic efficiencies than the parental enzyme (Caniça *et al.*, 1998).

Besides ESBL and IRT enzymes, there are some TEM family enzymes which possess these two types of phenotype simultaneously. The combination of substitutions associated with ESBL phenotypes and substitutions related with IRT phenotypes form a group known as complex mutant of TEM enzymes (CMT) (Table 1.5).

First described in France in 1997 (Sirot *et al.*, 1997), CMT-1, also known as TEM-50, presented the ESBL substitutions of TEM-15 and the IRT-substitutions of IRT-4. However, when compared to these enzymes, CMT-1 presented lower level of resistance to ceftazidime and to amoxicillin plus clavulanic acid than TEM-15 and IRT-4, respectively (Sirot *et al.*, 1997). Other enzymes presenting amino acid substitutions of ESBL and IRT enzymes were also described in Poland and other regions of France (Fiett *et al.*, 2000; Poirel *et al.*, 2004; Robin *et al.*, 2007). Overall, CMT enzymes tend to maintain the level of resistance of ESBL or IRT enzymes, but often it is possible to verify the decrease of resistance towards one or both types of phenotypes.

The emergence of CMT enzymes, possibly associated with the clinical use of third generation cephalosporins and β-lactamase inhibitors, constitutes an alarming factor in β-lactamase evolution towards resistance to β-lactam antibiotics.

Table 1.5 - TEM enzymes presenting amino acid substitutions associated with CMT phenotype (according to website http://lahey.org/studies/temtable.asp)

β-Lactamase (Secondary names)	Amino Acid at position																	pI[a]
	6	16	39	69	104	130	164	165	182	237	238	240	244	265	275	276	284	
TEM-1	Gln	Phe	Gln	Met	Glu	Ser	Arg	Trp	Met	Ala	Gly	Glu	Arg	Thr	Arg	Asn	Ala	5.4
TEM-2			Lys															5.6
TEM-50 (CMT-1)				Leu	Lys						Ser					Asp		5.6
TEM-68 (CMT-2)											Ser	Lys		Met	Leu			5.7
TEM-89 (CMT-3)			Lys		Lys	Gly					Ser							6.28
TEM-109 (CMT-5)				Leu	Lys		His											6.0
TEM-121 (CMT-4)			Lys		Lys		Ser			Thr		Lys	Ser					nd
TEM-123	Lys				Lys						Ser				Ala			nd
TEM-124	Lys				Lys				Thr						Ala			nd
TEM-125 (CMT-type)		Leu		Leu			Ser	Arg								Asp		5.2
TEM-151 (CMT-type)				Val			His									Asp	Gly	nd
TEM-152 (CMT-type)				Val			His					Lys				Asp		nd
TEM-158 (CMT-9)				Leu			Ser									Asp		nd

[a] Isoeletric point.
nd, not determined.

3.3.3. SHV β-lactamases

SHV enzymes are classified in groups 2b and 2be of the Bush-Jacoby-Medeiros classification scheme and in Ambler class A, and they are constitutive of the majority of *K. pneumoniae* strains which possess chromosomal-mediated β-lactamases. Once believed to be ubiquitous of this species, SHV-1 (sulphydryl reagent variable) confers resistance to ampicillin, amoxicillin, carbenilcillin and ticarcillin (Babini & Livermore, 2000; Livermore, 1995). First called PIT-2 (from the author's name Pitton), this enzyme was also detected in other *Enterobacteriaceae* as a plasmid-mediated β-lactamases (Matthew, 1979; Pitton, 1972).

The great majority of *K. pneumoniae* strains possesses a chromosomal copy of either bla_{SHV-1} or bla_{SHV-11} or close relatives, encoding non-extended-spectrum enzymes (Chaves *et al.*, 2001; Lee *et al.*, 2006b). Plasmid-mediated bla_{SHV} genes are possibly descendent from genome to plasmid mobilization events mediated by IS*26* (Ford & Avison, 2004). One such event resulted in an IS*26* insertion 2 kbp upstream of the bla_{SHV} coding sequence, such as it can be seen in the plasmid-mediated SHV-5 enzyme (Gutmann *et al.*, 1995). Another event resulted in IS*26* insertion into the bla_{SHV} promoter as can be identified in plasmid-mediated SHV-2a, SHV-11 and SHV-12 (Nüesch-Inderbinen *et al.*, 1997; Podbielski *et al.*, 1991b). Furthermore, some studies suggested that this IS*26* insertion increases promoter strength through the introduction of a different –35 region (Podbielski *et al.*, 1991a). Other studies demonstrated the importance of plasmid-mediated non-ESBL-encoding bla_{SHV} genes as percussors to ESBL-encoding genes (Hammond *et al.*, 2008; Lee *et al.*, 2006b).

Several studies have showed that the hyperproduction of SHV-1 enzymes associated or not with the impermeability mechanism of resistance, could lead to the false identification of ESBL production, such as the case of TEM-1 (Fu *et al.*, 2007; Miró *et al.*, 1998; Wu *et al.*, 2001). Overall, over 110 SHV β-lactamases have now been identified, according to the Lahey Clinic internet server (http://lahey.org/studies) (Table 1.6). In contrast to the TEM family, the vast majority of SHV enzymes are penicillinases and only 33 enzymes possess amino acid substitutions which confer an ESBL or IRS (inhibitor resistance SHV) phenotype.

Phylogenetic studies suggest that TEM and SHV enzymes diverged from each other 300-400 million years ago (Hall & Barlow, 2004). Furthermore, SHV probably evolved in the antibiotic era from an as-yet-unidentified common ancestor. These descendants include both penicillinases and extended-spectrum β-lactamases (Hall & Barlow, 2004). Sharing 67% of sequence identity with TEM enzymes, the SHV-1 substrate binding cavity is 0.7-1.2 Å larger than in TEM-1 (Kuzin *et al.*, 1999; Reynolds *et al.*, 2006).

Table 1.6 - SHV enzymes presenting amino acid substitutions associated with ESBL phenotype (according to website http://lahey.org/studies)

β-Lactamase	Amino acid at position																				pI[a]
	8	35	43	48	54	64	75	89	122	140	156	158	173	179	192	193	195	205	238	240	268	
SHV-1	Ile	Leu	Arg	Glu	Gly	Glu	Val	Glu	Leu	Ala	Gly	Asn	Leu	Asp	Lys	Leu	Thr	Arg	Gly	Glu	Thr	7.6
SHV-2																			Ser			7.6
SHV-2[a]		Gln																	Ser			7.6
SHV-3																		Leu	Ser			7.0
SHV-4																		Leu	Ser	Lys		7.8
SHV-5																			Ser	Lys		8.2
SHV-6														Ala								7.6
SHV-7	Phe		Ser																Ser	Lys		7.6
SHV-8														Asn								7.6
SHV-9					Del					Arg					Asn	Val			Ser	Lys		8.2
SHV-12		Gln																	Ser	Lys		8.2
SHV-13		Gln																	Ala			7.6
SHV-15		Gln		Lys			Met	Lys											Ser	Lys		nd
SHV-18	Phe		Ser																Ala	Lys		7.8
SHV-20													Phe						Ser			7.6
SHV-21									Phe				Phe						Ser			nd
SHV-22												Lys							Ser	Lys		7.6
SHV-24														Gly								nd
SHV-29		Gln	Ser																Ala			nd
SHV-30	Phe		Ser																Ser			nd
SHV-31		Gln																		Lys		nd
SHV-34	Phe		Ser			Gly													Ser			nd
SHV-39																			Ser		Ser	nd
SHV-45											Asp								Ser	Lys		8.2
SHV-46																	Asn		Ser	Lys		nd
SHV-64		Gln					Leu												Ser	Lys		nd
SHV-66		Gln				Gln													Ser	Lys		nd
SHV-86		Gln																	Ser	Arg		nd
SHV-97																				Lys	Ser	nd
SHV-102																			Ala			nd

[a] Isoeletric point.
nd, not determined.

SHV β-lactamases may present amino acid substitutions which can be responsible for increasing the levels of resistance to narrow and extended-spectrum cephalosporins and monobactams, as well as to β-lactamase inhibitors. Amino acid substitutions in residues Asp179, Gly238 and Glu240 have been found in clinical strains presenting resistance to third generation cephalosporins (Table 1.6). Studies based on site saturation mutagenesis for residue Asp104 show that, just like in TEM, this amino acid plays an important role in substrate binding and recognition of oxyimino-cephalosporins (Bethel *et al.*, 2006).

Residue Asp179, also associated to the ESBL phenotype, is located in the Ω loop of the SHV active site. Amino acid substitution of Asp179 by Ala (SHV-6), Asn (SHV-8) and Gly (SHV-24) possibly increase the movement of the Ω loop, expanding the binding site and destabilizing the Glu166 (Knox, 1995). This substitution conferred high-level resistance to ceftazidime but not to cefotaxime in enzymes isolated in France (SHV-6), Japan (SHV-24) and United States (SHV-8) (Arlet *et al.*, 1997; Kurokawa *et al.*, 2000; Rasheed *et al.*, 1997).

In contrast to the TEM enzymes, SHV β-lactamase substitutions of the residue Gly238 are of greater importance in conferring high-level resistance against extended-spectrum cephalosporins. As mentioned above, the Gly238Ser pushes the β-strand out and away from the reactive Ser70, which expands the active site even more when compared to TEM, and permits greater substrate versatility against penicillins and cephalosporins (Hujer *et al.*, 2001). Other site-directed mutagenesis studies corroborate these findings (Huletsky *et al.*, 1993; Hujer *et al.*, 2002).

Enzymes such as SHV-2, SHV-2A and SHV-3, among others, confer higher level of resistance to cefotaxime than to ceftazidime (Barthélemy *et al.*, 1988; Nicolas *et al.*, 1989; Podbielski *et al.*, 1991b). Once again, just like in TEM enzymes, the amino acid substitution of residue Glu240 increases the hydrolysing activity against ceftazidime. Resistance to this antibiotic is increased in enzymes such as SHV-5 and SHV-12, which possess at least both Gly238Ser and Glu240Lys substitutions (Gutmann *et al.*, 1995; Nüesch-Inderbinen *et al.*, 1997). Other residues, like Thr182 that occurs naturally in SHV enzymes may stabilize the overall structure of the proteins (Huang & Palzkill, 1997; Wang *et al.*, 2002).

Contrary to what happens in TEM β-lactamases, there are few SHV that present resistance to β-lactamase inhibitors. Until now, only four enzymes were detected in Europe and Taiwan which present mutations that can confer loss of susceptibility to these antibiotics. Amino acid substitutions of residues Met69 and Ser130 confer an increase of resistance against clavulanic acid associated with the loss of affinity to penicillins and narrow-spectrum cephalosporins, just like in TEM enzymes (Dubois *et al.*, 2004; Prinarakis *et al.*; 1997). While Met69 has been detected in SHV-49 and SHV-92 (Table 1.7), Ser130 was detected in SHV-

10 and was associated with the high decrease of activity against extended-spectrum β-lactamases (Prinarakis *et al.*; 1997).

A different amino acid substitution, Ala187Thr, was also associated with low level resistance to β-lactamase inhibitors in SHV-26 (Chang *et al.*, 2001). However, the Ala187 residue is located relatively far from the active site and the authors do not explain the molecular mechanism implied in it. Other SHV mutants were constructed *in vitro* by site-saturation mutagenesis based in the observations of the effect of specific amino acid substitutions in positions 166, 244 and 276 of naturaly occuring TEM enzymes (Padayatti *et al.*, 2005; Randegger & Hächler, 2001a; Thomson *et al.*, 2006). In an attemp to construct complex mutant SHV enzymes, with amino acid substitutions involved in ESBL and IRS phenotypes, it was concluded that SHV enzymes do no benefit proportionally from the simultaneous presence of amino acid substitutions in residues associated with these phenotypes (Randegger & Hächler, 2001a).

Table 1.7 - SHV enzymes presenting amino acid substitutions associated with IRS phenotype (according to website http://lahey.org/studies)

β-Lactamase	Amino acid at position												pI[a]
	35	54	69	112	130	140	141	187	192	193	238	240	
SHV-1	Leu	Gly	Met	His	Ser	Ala	Thr	Ala	Lys	Leu	Gly	Glu	7.6
SHV-10		Del			Gly	Arg			Asn	Vel	Ser	Lys	8.2
SHV-26								Thr					7.6
SHV-49			Ile										nd
SHV-92	Gln		Ile				Ile						nd

[a] Isoeletric point.
nd, not determined.

According to the identification of amino acid substitutions that confer resistance to extended-spectrum cephalosporins and/or to β-lactamase inhibitors, more than 53 SHV β-lactamases are penicillinases, while TEM family present no more than 20 enzymes of this type. Nevertheless, the lesser number of ESBL-encoding SHV enzymes is distributed worldwide, such as the case of TEM enzymes. Enzymes like SHV-2, SHV-2A, SHV-5 and SHV-12 have been detected in strains of *Enterobacteriaceae*, *Pseudomonadaceae* and *Moraxellaceae* isolated in hospitals, in the community, animals and food samples from Europe, Africa, Asia and Oceania (Chiaretto *et al.*, 2008; Damjanova *et al.*, 2007; Jouini *et al.*, 2007; Paterson & Bonomo, 2005).

3.3.4. LEN and OKP β-lactamases

Instead of SHV enzymes, *K. pneumoniae* strains can express two other types of enzymes, whose encoding genes may be located in the chromosome. These two other β-

lactamases are LEN (from *K. pneumoniae* strain LEN-1) enzymes and OKP (other *K. pneumoniae* β-lactamase) enzymes (Arakawa *et al.*, 1986; Hæggman *et al.*, 2004), both also conferring resistance to ampicillin.

According to phylogenetic studies performed with *gyrA*, *K. pneumoniae* strains can be distributed in three clusters: groups KpI, KpII and KpIII, which express SHV, OKP and LEN enzymes, respectively (Brisse & Verhoef, 2001; Hæggman *et al.*, 2004). Studies performed in *K. pneumoniae* strains identified more than 80% of strains as belonging to group KpI, while groups KpII and KpIII were equally represented with 10% each (Brisse & Verhoef, 2001; Fu *et al.*, 2007). Hæggman *et al.* (2004) estimated the time of divergence of the phylogenetic groups KpI and KpIII as occurring between 6 and 28 million years ago.

The OKP family, found in group KpII, is more heterogeneous than the SHV and LEN families, presenting 88-89% similarity to SHV-1 and LEN-1. More recently, the *K. pneumoniae* phylogenetic group KpII was sub-divided into two subgroups, KpII-A and KpII-B, each one expressing an OKP type enzyme (Fèvre *et al.*, 2005). With 95% of similarity between them, OKP-A and OKP-B are expressed by strains belonging to subgroups KpII-A and KpII-B, respectively.

All of these β-lactamases are usually produced at low levels, conferring resistance to ampicillin, amoxicillin, carbenicillin and ticarcillin, with MIC values of 32 to 256 µg/mL for enzyme producers. However, strains of *K. pneumoniae* which do not possess a constitutive enzyme, were reported with MICs values of 2 µg/mL to ampicillin and to ticarcillin, which constitute extremely rare phenotypes (Livermore, 1995). Overall, LEN and OKP enzymes have been detected in America, Europe and Japan, but only in sporadic cases (Hæggman *et al.*, 2004; Fèvre *et al.*, 2005; Arakawa *et al.*, 1986; Melano *et al.*, 2006).

3.3.5. KLUA, KLUC and KLUG β-lactamases

The enterobacterial *Kluyvera* genus is composed of four species, *Kluyvera ascorbata*, *Kluyvera cryocrescens*, *Kluyvera georgiana*, and *Kluyvera cochleae* and presents a chromosomally encoded class A β-lactamase in at least three of them (Decousser *et al.*, 2001; Farmer, 1999; Humeniuk *et al.*, 2002, Poirel *et al.*, 2002c). Although phylogenetically related, *Kluyvera* species produce different β-lactamases sharing similar substrate profiles. The KLUA-1 β-lactamase confers low level resistance to penicillins, cephalothin and cefuroxime, and is inhibited by clavulanic acid, while KLUC-1, in the natural strain, confers resistance to amoxicillin and ticarcillin and reduced susceptibility to cephalothin and cefuroxime (Decousser *et al.*, 2001; Humeniuk *et al.*, 2002). Once cloned, this enzyme conferred resistance or reduced susceptibility to cefotaxime, ceftriaxone, cefpirome and

aztreonam. A *K. georgiana* strain producing KLUG-1 presented intermediate susceptibility to amoxicillin, ticarcillin and cephalothin and, once cloned, KLUG-1 also conferred resistance or reduced susceptibility to piperacillin, cefotaxime, ceftriaxone, cefepime, cefpirome and aztreonam, which is the same profile as that which is observed for plasmid-mediated CTX-M-8 (Bonnet *et al.*, 2000b; Poirel *et al.*, 2002c).

Chromosomal encoded β-lactamases from *Kluyvera* spp. seem to be closely related with rapidly expanding plasmid-mediated CTX-M (active on cefotaxime, first isolated at Munich) enzymes (Bauernfeind *et al.*, 1990). Overall, KLUC-1 shares only 86% similarity with CTX-M-1, while KLUA-1 and KLUG-1 shares 99% similarity with CTX-M-2 and CTX-M-8, respectively. Considered as probable progenitors of specific sub-groups of CTX-M enzymes, *Klyuvera* β-lactamase encoding genes present upstream and downstream regions with high similarity with genes encoding for CTX-M enzymes (Decousser *et al.*, 2001; Humeniuk *et al.*, 2002, Poirel *et al.*, 2002c). Mobilization elements such as IS*Ecp1* and IS*10*-like have been identified upstream of $bla_{\text{CTX-M}}$ and of $bla_{\text{KLU-type}}$ genes, increasing the relationship between the two families (Bonnet *et al.*, 2000b; Karim *et al.*, 2001).

3.3.6. CTX-M β-lactamases

Enzymes of the CTX-M family have become, in the past decade, the most prevalent ESBL, both in nosocomial and community settings (Cantón & Coque, 2006). Initially reported in the second half of the 1980s, their rate of dissemination among bacteria and in most parts of the world has increased dramatically since 1995. First detected in Japan in 1986 and later designated as CTX-M-1 in 1989 in Germany, the rapidly growing CTX-M family of β-lactamases now consists of more than 80 members (Bauernfeind *et al.*, 1990; Matsumoto *et al.*, 1988).

CTX-M β-lactamases divide into five major groups based on sequence homology: the CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 groups (http://www.lahey.org/studies/webt.stm) (Table 1.8). Members of each group share more than 94% identity, and members of different groups less than 90% (Bonnet, 2004). According to phylogenetic studies, the CTX-M-1 group includes widely disseminated enzymes like CTX-M-1, CTX-M-3 and CTX-M-15, while the CTX-M-2 group includes less disseminated enzymes like CTX-M-2, CTX-M-4 and CTX-M-6. The also highly disseminated CTX-M-9 group consists of enzymes like CTX-M-9 and CTX-M-14 (Cantón & Coque, 2006).

Table 1.8 - Different CTX-M clusters and origin of *bla*$_{CTX-M}$ (adapted from Cantón & Coque, 2006)

CTX-M Group	Year (enzyme, country)[a]	Enzymes	Origin
CTX-M-1	1989 (CTX-M-1, Germany)	CTX-M-1, -3, -10, -11, -12, -15, -22, -23 -29, -30, -32, -33, -28, -36, -54, UOE-1	*K. ascorbata*
CTX-M-2	1986 (FEC-1, Japan)	CTX-M-2, -4, -6, -7, -20, -31, -44 (previously TOHO-1), FEC-1	*K. ascorbata*
CTX-M-8	1996 (CTX-M-8, Brazil)	CTX-M-8, -40	*K. georgiana*
CTX-M-9	1994 (CTX-M-9, Spain)	CTX-M-9, -13, -14, -16, -17, -18, -19, -24, -27, -45 (previously TOHO-2), -46; -47, -48, -49, -50	*K. georgiana*
CTX-M-25	2000 (CTX-M-25, Canada)	CTX-M, -26, -25, -39, -41	ND

[a] Year of first isolation or description (first enzyme described and country of isolation); CTX-M-14 and CTX-M-18 are identical. ND, not defined.

Enzymes from the CTX-M-1 and CTX-M-2 groups may have evolved from the *Kluyvera ascorbata* chromosomal genes *bla*$_{KLUA}$, while other studies suggest that enzymes from the CTX-M-8 and CTX-M-9 groups derived from the *Kluyvera georgiana* chromosomal gene, *bla*$_{KLUG}$ (Olson *et al.*, 2005; Rodriguez *et al.*, 2004). Futhermore, several studies prove that the neighbouring sequences of the *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, and *bla*$_{CTX-M-10}$ genes exhibit high nucleotide identity with those surrounding *Kluyvera* chromosomal β-lactamase genes (Oliver *et al.*, 2001; Olson *et al.*, 2005; Power *et al.*, 2005).

Plasmids encoding CTX-M enzymes are often highly transmissible, a property that can explain the easy dissemination of *bla*$_{CTX-M}$-harbouring plasmids (Baraniak *et al.*, 2002b). However, in the case of non-self-transmissible plasmids harbouring *bla*$_{CTX-M}$ genes, transfer can still occur by transformation or by the influence of coexisting conjugative plasmids (Cao *et al.*, 2002; Tassios *et al.*, 1999). High identities between the regions surrounding *K. georgiana* chromosomal *bla*$_{KLUG}$ genes or the *K. ascorbata* chromossonal *bla*$_{KLUA}$ gene and the regions surrounding the plasmid-mediated *bla*$_{CTX-M-8}$ gene or genes belonging to CTX-M-2 group suggest the mobilization of this type of β-lactamase-encoding gene from the chromosome to plasmids (Humeniuk *et al.*, 2002; Poirel *et al.*, 2002c). This process could explain the later expansion of CTX-M enzymes, in opposition to TEM and SHV ESBLs, which derived from widespread plasmid-mediated enzymes.

Among the elements that are involved in the mobilization of *bla*$_{CTX-M}$ genes, insertion sequences (IS, e.g., IS*Ecp1* or IS*Ecp1*-like) have repeatedly been observed upstream of ORFs encoding the several *bla*$_{CTX-M}$ genes (Perez *et al.*, 2007). Some studies have suggested that the IS*Ecp1* element not only confers mobility to *bla*$_{CTX-M}$ genes, but also probably provides the promoter for expression of these genes (Cao *et al.*, 2002; Karim *et al.*, 2001; Poirel *et al.*, 2005). A specific sequence located downstream of *Orf513* has been proposed to be a recognition site for the *Orf513* putative recombinase. A 17-bp fragment of

this sequence has been identified downstream of certain IS*Ecp1* sequences (Saladin *et al.*, 2002), suggesting a complex mobilization process involving the *Orf513* putative recombinase and the IS*Ecp1* insertion sequence.

Other CTX-M-encoding genes have been observed in class 1 integrons, like $bla_{CTX\text{-}M\text{-}2}$ (Arduino *et al.*, 2002). This gene is located in a complex *sul1*-type integron, termed In35 which includes *Orf513*. It is suggested that the $bla_{CTX\text{-}M\text{-}2}$ gene, which shares high homology with the chromosomal $bla_{KLUA\text{-}1}$ gene, was possibly acquired by a plasmid through an uncharacterised recombinational event in which *Orf513*, the so-called CR (common region) element, could be involved (Arduino *et al.*, 2002; Partridge & Hall, 2003). This new type of genetic element was recently identified as being closely associated with the spread of many antibiotic resistance genes (Toleman *et al.*, 2006). ISCR elements can be divided into two groups: ISCRs1 are those that form complex class 1 integrons and ISCRs-2 to -13 are those associated with non-class 1 integrons (Bebrone, 2007). Furthermore, a study based on multiple clinical isolates, identified the IS*CR1* next to $bla_{CTX\text{-}M\text{-}2}$ in all $bla_{CTX\text{-}M\text{-}2}$-containing Gram-negative isolates (Arduino *et al.*, 2003). The authors suggested that this IS element is strongly associated with the emergence and dissemination of the $bla_{CTX\text{-}M\text{-}2}$ gene in South America. In Europe, $bla_{CTX\text{-}M\text{-}9}$ is most commonly associated with IS*CR1* elements as part of a complex class 1 integron, In60 (Toleman *et al.*, 2006).

Mobile elements, such as IS*10* and IS*26,* have been observed upstream of $bla_{CTX\text{-}M\text{-}8}$ (Bonnet *et al.*, 2000b) and $bla_{CTX\text{-}M\text{-}1}$, respectively, while an IS*903*-like element was observed downstream of the $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}17}$ genes (Cao *et al.*, 2002; Pai *et al.*, 2001; Saladin *et al.*, 2002).

It is possible to detect isolates producing CTX-M enzymes all over the world, particulary CTX-M-15, CTX-M-14, CTX-M-3, CTX-M-2, CTX-M-9, among others (Figure 1.4). Although some countries present only sporadic reports of CTX-M producing isolates, other regions face endemic situations. CTX-M-2 was detected in Brazil and Argentina, among others CTX-M-type enzymes (Bonnet, 2004). Among community isolates from Bolivia and Peru, *E. coli* harbouring CTX-M-15 and CTX-M-9 were predominant (Pallecchi *et al.*, 2007). In the Far East, surveys conducted in Japan showed that CTX-M-2 and CTX-M-3 were predominant, while in China, CTX-M-3, CTX-M-13 and CTX-M-14 enzymes were already the most frequent ESBL in 1997 and 1998 (Chanawong *et al.*, 2002; Yagi *et al.*, 2000; Yamasaki *et al.*, 2003). In India, the most successful and widespread CTX-M-15 was detected in a two month period of the year 2000 and already in three different species of the *Enterobacteriaceae* family (*E. coli*, *K. pneumoniae* and *Enterobacter aerogenes*) (Karim *et al.*, 2001).

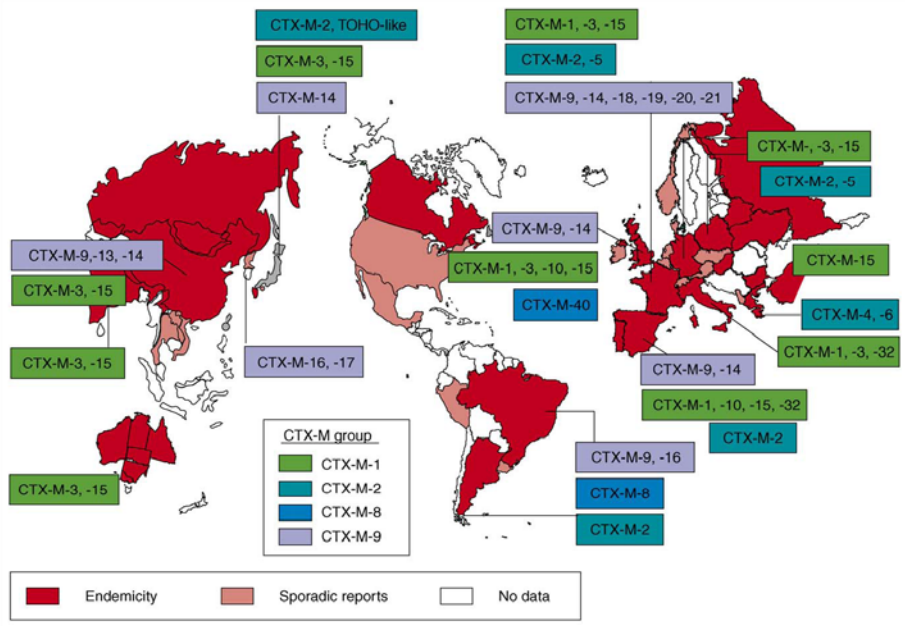

Figure 1.4 - Dominant and frequent CTX-M β-lactamase types in different global regions (adapted from Cantón & Coque, 2006).

In the United Kingdom, France, Spain and Italy, surveillance studies detected an increasing frequency of production of CTX-M-15 among clonally related *E. coli* strains (Brigante *et al.*, 2005; Lavigne *et al.*, 2007; Livermore & Hawkey, 2005; Mugnaiol *et al.*, 2006). Nevertheless, CTX-M-2, CTX-M-3, CTX-M-9 and CTX-M-14, among others, were also detected in Europe, but in a lesser frequency (Figure 1.5).

CTX-M enzymes can be detected both in community and hospital environments. Although initial observation of infections caused by bacteria harbouring CTX-M enzymes was in hospitals, they rapidly spread to the community. Nursing homes may serve as reservoirs from which colonized and infected patients transfer these ESBL-producing strains to the community or back to the hospitals (Pitout *et al.*, 2005; Wiener *et al.*, 1999). Some of the identified risk factors for infection with bacteria producing CTX-M are recent hospitalisation, age, and exposure to cephalosporins and/or quinolones (Perez *et al.*, 2007). The detection of CTX-M-producing bacteria in food-producing animals could indentify them as potential reservoirs of resistant bacteria that intersect with the community (Duan *et al.*, 2006; Meunier *et al.*, 2006).

CTX-M enzymes usually confer high-level resistance to aminopenicillins, carboxypenicillins, ureidopenicillins and narrow-spectrum cephalosporins, while retaining susceptibility to cefoxitin and carbapenems. Kinetic studies show that CTX-M enzymes are less effective against penicillins than TEM and SHV penicillinases, as for most class A ESBLs (Bonnet, 2004).

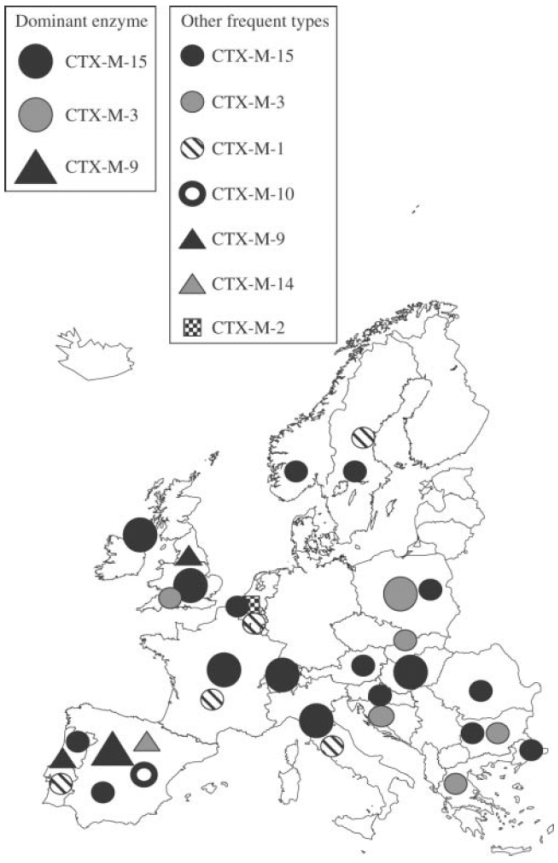

Figure 1.5 - Dominant and frequent CTX-M β-lactamase types in different European countries (adapted from Livermore *et al.*, 2007).

The enzymes of the CTX-M family are more active against cefotaxime and ceftriaxone than against ceftazidime. In contrast with TEM and SHV cefotaximases (e.g. TEM-3, TEM-4, SHV-2, and SHV-3), CTX-M enzymes present weaker enzymatic efficiencies. However, some point mutations can increase activity against ceftazidime. For example, CTX-M-15 and CTX-M-32 differ from CTX-M-3 and CTX-M-1, respectively, by an Asp240Gly substitution. Nevertheless, they present 100-fold more activity against ceftazidime than CTX-M-3 and CTX-M-1 (Cartelle *et al.*, 2004; Poirel *et al.*, 2002b). CTX-M-27 and CTX-M-16, differing from CTX-M-9 and CTX-M-14, respectively, by means of the same mutation, also present higher catalytic efficiencies against ceftazidime (Bonnet *et al.*, 2001; Bonnet *et al.*, 2003). Another substitution responsible for higher levels of resistance to ceftazidime, as compared to cefotaxime, is the Pro167Ser mutation, which differentiates CTX-M-19 from CTX-M-14 (Poirel *et al.*, 2001b). The same mutation has been observed in other β-lactamases, such as PSE-4 (*Psedomonas*-specific enzyme), with similar results (Therrien *et al.*, 1998). Overall, CTX-M enzymes show a great degree of susceptibility to β-lactamase

inhibitors, although a low level of resistance to the combination of clavulanic acid with amoxicillin and ticarcillin could be observed (Bonnet, 2004).

3.3.7. OXA β-lactamases

Classified in group 2d, OXA (active on oxacillin) enzymes are class D β-lactamases capable of hydrolysing isoxazolilpenicillins, such as oxacillin, and other penicillins (Bush *et al.*, 1995). Currently, over than 127 different variants of OXA enzymes have been identified on the protein level (http://lahey.org/studies).

Overall, there is a generally weak sequence identity between the various OXA-family members and even weaker homologies between them and Class A and C enzymes. However, all three classes share the same topological fold and hydrolyze substrates by a similar serine nucleophile-mediated acylation/deacylation mechanism (Massova & Mobashery, 1998). OXA-1, the most common type in enterobacteria (Liu *et al.*, 1992), generally confers low level resistance to penicillins but not to broad-spectrum β-lactams, and some producers of this enzyme are resistant to clavulanic acid, but not to piperacillin-tazobactam combination (Wu *et al.*, 1992). Although the bla_{OXA-1} gene shows more than 50% divergence when compared with bla_{OXA-2}, there is a functional similarity at the peptide level (Ouellette *et al.*, 1987). When comparing OXA-2 with OXA-3, it is possible to detect 95% homology between them, which confers similar proprieties (Sanschagrin *et al.*, 1995). Similar phenotypes are produced by OXA-1 and OXA-4, the latter differing from OXA-1 in two aminoacids (Asp48Val and Asp207Glu) (Philippon *et al.*, 1986).

Most OXA-type β-lactamases are not regarded as ESBLs, because they do not hydrolyse the extended-spectrum cephalosporins. However, OXA-10, first called PSE-2 in *Pseudomonas aeruginosa* (Huovinen *et al.*, 1988), can weakly hydrolyse cefotaxime, ceftriaxone and aztreonam (Hall *et al.*, 1993). Other OXA ESBL enzymes that confer resistance to cefotaxime, and sometimes to ceftazidime and aztreonam, are already identified, as OXA-11, OXA-14 to OXA-19, OXA-28, OXA-31, OXA-32, OXA-45 (Aubert *et al.*, 2001; Danel *et al.*, 1995; Danel *et al.*, 1997; Danel *et al.*, 1998; Danel *et al.*, 1999; Hall *et al.*, 1993; Mugnier *et al.*, 1998; Philippon *et al.*, 1997; Poirel *et al.*, 2001a; Poirel *et al.*, 2002a; Toleman *et al.*, 2003). Mostly detected in Europe, there are very few epidemiological data on the geographical spread of OXA-type ESBLs. An aminoacid substitution at position 167 (Gly167Asp) increases resistance to ceftazidime, as detected for OXA-11, OXA-14, OXA-16 and OXA-19 (Hall *et al.*, 1993; Danel *et al.*, 1995; Danel *et al.*, 1998; Mugnier, 1998) (Table 1.9). Another aminoacid substitution associated with the increase of resistance to ceftazidime was detected in position 164 (Trp164Gly) at OXA-28 (Poirel *et al.*, 2001a).

Table 1.9 - Extended-Spectrum β-Lactamases closely related with OXA-10 (according to http://lahey.org/studies)

β-lactamase	Amino acid at position													pI[a]
	10	20	58	76	110	127	144	164	167	184	240	258	272	
OXA-10	Ile	Gly	Asp	Asn	Thr	Ala	Tyr	Trp	Gly	Tyr	Glu	Ser	Glu	6.1
OXA-11							Ser		Asp					6.4
OXA-14									Asp					6.2
OXA-16						Thr			Asp					6.2
OXA-17				Ser										6.1
OXA-19	Thr	Ser	Asn		Ser				Asp	Phe	Gly	Asn	Ala	7.5
OXA-28	Thr	Ser	Asn		Ser			Gly		Phe	Gly	Asn	Ala	8.1

[a] Isoelectric point.

OXA-15, which is derived from OXA-2, presents an aminoacid substitution also capable of conferring resistance to ceftazidime (Asp149Gly) (Danel *et al.*, 1997) (Table 1.10). OXA-32, also derived from OXA-2, presents a Leu169Ile substitution capable of conferring resistance to ceftazidime, but not to cefotaxime (Poirel *et al.*, 2002a).

OXA-31 is related to OXA-1, also called OXA-30, and to its derivative OXA-4 (Boyd & Mulvey, 2006; Siu *et al.*, 2000); OXA-31 differs from them in four and three aminoacid substitutions, respectively (Ouellette *et al.*, 1987; Sanshagrin *et al.*, 1995). All of these three enzymes confer similar phenotypes, as resistance to cefepime and susceptibility to ceftazidime. They may selectively hydrolyze some 2-amino-5-thiazolyl cephalosporins (cefpirome, cefepime, and cefclidin) but not others (ceftazidime, cefotaxime), these drugs mostly differing by substitutions at the C-3 of the cephem core (Aubert *et al.*, 2001).

Table 1.10 - Extended-Spectrum β-Lactamases closely related with OXA-2 (according to http://lahey.org/studies)

β-lactamase	Amino acid at position		pI[a]
	149	169	
OXA-2	Asp	Leu	7.7
OXA-15	Gly		8.0
OXA-32		Ile	7.7

[a] Isoelectric point.

OXA-18 and OXA-45 are members of the novel subgroup 2d', related to other group 2d β-lactamases, but with extended-spectrum hydrolytic properties and full inhibition by clavulanic acid (Philippon *et al.*, 1997; Toleman *et al.*, 2003).

From the 127 enzymes identified, at least 45 exhibit carbapenem-hydrolysing activity, and they can be subclassified into eight subgroups (Walther-Rasmussen & Høiby, 2006)

(Table 1.11). The majority of carbapenem-hydrolysing OXA enzymes are detected in the *Acinetobacter baumannii* chromosome (Table 1.11).

Table 1.11 - Compilation of the OXA-type carbapenemase-producing bacteria and geographicaly distribution (adapted from Walther-Rasmussen & Høiby, 2006)

Subgroup	Enzyme	Genetic location[a]	Host	Country
I	OXA-23	C/(C)/P	Mainly *Acinetobacter baumannii*	Countries from different regions
	OXA-27	(C)	*A. baumannii*	Singapore
	OXA-49		*A. baumannii*	China
II	OXA-24	(C)	*A. baumannii*	Spain
	OXA-25	(C)	*A. baumannii*	Spain
	OXA-26	(C)	*A. baumannii*	Belgium
	OXA-40	(C)	*A. baumannii*	France
	OXA-72		*A. baumannii*	Thailand
III	OXA-51	C	*A. baumannii*	Argentina
	OXA-64	C	*A. baumannii*	Rep. of South Africa
	OXA-65	C	*A. baumannii*	Argentina
	OXA-66	C	*A. baumannii*	Spain
	OXA-68	C	*A. baumannii*	Spain
	OXA-69	C	*A. baumannii*	Turkey
	OXA-70	C	*A. baumannii*	Hong Kong
	OXA-71	C	*A. baumannii*	Rep. of South Africa
	OXA-75	C	*A. baumannii*	France
	OXA-76	C	*A. baumannii*	France
	OXA-77	C	*A. baumannii*	na
	OXA-78	(C)	*A. baumannii*	Turkey?
IV	OXA-58	C	*A. baumannii*	France
V	OXA-55	C	*Shewanella algae*	France
	OXA-SHE	C	*S. algae*	na
VI	OXA-48	P	*K. pneumoniae*	Turkey
	OXA-54	C	*Shewanella oneidensis*	na
VII	OXA-50	C	*P. aeruginosas*	Countries from different regions
VIII	OXA-60	C	Raistonia pickettii	France

[a] C, chromosome; ©, probably a chromosome; P, plasmid.
na, not available.

The sequence identities between members of each subgroup are more than 92.5%, while it ranges from 40 to 70% between enzymes from different subgroups. OXA-type carbapenemases present a diverse substrate range, but generally these enzymes hydrolyse penicillins and narrow-spectrum cephalosporins efficiently, while extended-spectrum cephalosporins are only very poorly hydrolysed, if at all (Walther-Rasmussen & Høiby, 2006). While in the late 1990s, carbapenems were the only remaining useful agent against severe *Acinetobacter* infections, natural selection or insertion into the chromosome by recombination, co-integration or transposition of the carbapenems resistance genes, has

now transformed this genus into one of the major producers of class D enzymes (Livermore & Woodford, 2006; Walther-Rasmussen & Høiby, 2006).

A study conducted in 2002 (Barlow & Hall, 2002) concluded that the class D OXA enzymes were limited to Gram-negative bacteria, but some homologes of the OXA genes were found in the chromosome of Gram-positive bacteria, suggesting a horizontal transfer into these type of bacteria occurring between 575 and 520 million years ago. Additional horizontal transfer events within the Gram-negative bacteria were also apparent. The phylogeny shows that the OXA genes have been mobilized to plasmids about 116 ± 25 million years ago and again 42 ± 9 million years ago, with a third mobilization occurring too recently to estimate accurately (Barlow & Hall, 2002). This is an indication that mobilization to plasmids and horizontal transfer is a phenomenon that predates the use of antibiotics (Hall & Barlow, 2004).

Regarding OXA-type carbapenemases, a possible origin for these enzymes can be found on the soil. Imipenem is a derivative of thienamycin, which is a natural product of the soil organism *Streptomyces cattleya* (Kahan *et al.*, 1979). Thus, possession of carbapenem-hydrolysing enzymes would be beneficial for other soil bacteria. Other organisms may have developed enzymes with carbapenemase activity or acquired carbapenemase-encoding genes by means of conjugative plasmids (Walther-Rasmussen & Høiby, 2006).

3.3.8. Metallo-β-lactamases

Class B β-lactamases, or metallo-β-lactamases, required one or two zinc ions for their activity. This type of enzymes can degrade all classes of β-lactams except monobactams, but they are of particular interest because of their high capacity to hydrolyse carbapenems. Metallo-β-lactamases, like all β-lactamases, can be divided into those that are normally chromosomally mediated and those that are encoded by transferable genes (Walsh *et al.*, 2005) (Table 1.12).

Class B β-lactamases can be subdivided into three subclasses, B1 to B3, according to sequence alignments (Garau *et al.*, 2004). Subclass B1 β-lactamases share more than 23% identity, while members of subclass B2 present 11% identity with subclass B1 members. Subclasses B1 and B3 hydrolyze most β-lactam antibiotics including carbapenems. Aztreonam is not hydrolysed by these enzymes, which are not inhibited by β-lactamase inhibitors. Enzymes belonging to subclass B2 efficiently hydrolyze only carbapenems (Bebrone, 2007). Phylogenetic studies suggest that B1 and B2 descend from a common ancestor and subclass B3 shares only structural similarities with these subclasses (Garau *et al.*, 2005).

Table 1.12 - Metallo-β-lactamases divided into three subclasses and classified by year of discovery. Not all variants are included in this table (adapted from Bebrone, 2007)

Subclass	Enzyme	Strain	Year of Discovery	Structure
B1	BcII	*Bacillus cereus*	1966	Mono-zinc, Di-zinc Apo-form
	Bce 170	Alkalophilic *Bacillus* spp.	1985	
	CcrA	*Bacillus fragilis*	1990	Di-zinc
	BlaB	*Elizabethkingia meningoseptica*	1998	Di-zinc
	IND-1	*Chryseobacterium indologenes*	1999	
	CGB-1	*Chryseobacterium gleum*	2002	
	EBR-1	*Elizabethkingia brevis*	2002	
	MUS-1	*Myroides odoratimimus*	2002	
	TUS-1	*Myroides odoratus*	2002	
	Bla2	*Bacillus anthracis*	2003	
	JOHN-1	*Flavobacterium johnsoniae*	2003	
	SFB-1	*Shewanella frigidimarina*	2005	
	SLB-1	*Shewanella livingstonensis*	2005	
Acquired B1	IMP-1	*Serratia marcescens, P. aeruginosa*	1994	Di-zinc
	VIM-1	*P. aeruginosa, A. baumannii*	1999	
	VIM-2	*P. aeruginosa, A. baumannii*	2000	Mono-zinc 1KO2, Di-zinc 1KO3
	IMP-2	*A. baumannii, S. marcescens*	2000	
	SPM-1	*P. aeruginosa*	2002	Mono-zinc
	VIM-4	*P. aeruginosa, K. pneumoniae, E. cloacae*	2003	
	GIM-1	*P. aeruginosa*	2004	
	SIM-1	*A. baumannii*	2005	
B2	CphA	*Aeromonas hydrophila*	1991	Mono-zinc
	ImiS	*A. veronii*	1996	
	Sfh-1	*S. fonticola*	2003	
B3	L1	*Stenotrophomonas maltophilia*	1991	Di-zinc, Mono-zinc 2H6A, Apo-form 2FU6
	GOB-1	*E. meningoseptica*	2000	
	FEZ-1	*Legionella gormanii*	2000	Di-zinc
	THIN-B	*Janthinobacterium lividum*	2001	
	Mbl1b	*Chryseobacterium crescentus*	2001	
	CAU-1	*Caulobacter vibrioides*	2002	
	BJP-1	*Bacillus japonicum*	2006	Di-zinc 2GMN

Among the transferable B1 metallo-β-lactamases, IMP- (active on Imipenem) and VIM-types (Verona integron-encoded metallo-β-lactamase) occur most frequently and the majority of genes encoding IMP- and VIM-type, as well as GIM-1(German imipenemase), are found as gene cassettes in class 1 or class 3 integrons (Walsh *et al.*, 2005). Another metallo-β-lactamase gene, bla_{SPM-1} is associated with the ISCR variant, ISCR4 (Bebrone, 2007). These enzymes have now spread worldwide (Figure 1.6).

IMP-1 was discovered in 1998 in Japan and constitutes, the most common metallo-β-lactamase in this country, having been discovered in several types of Gram-negative bacteria (Walsh *et al.*, 2005).

Overall, more than 18 IMP-type enzymes have now been described, mostly in Japan, China and Taiwan, among other countries. The increasing use of carbapenems in these countries may have led to the emergence of IMP metallo-β-lactamases (Helfand & Bonomo, 2003).

Among the VIM enzymes, the second most dominant group of transferable metallo-β-lactamases, the great majority of variants have been detected in Europe. First discovered in France in 2000, VIM-2 is currently the most widespread transferable metallo-β-lactamase (Poirel *et al.*, 2000) (Figure 1.6).

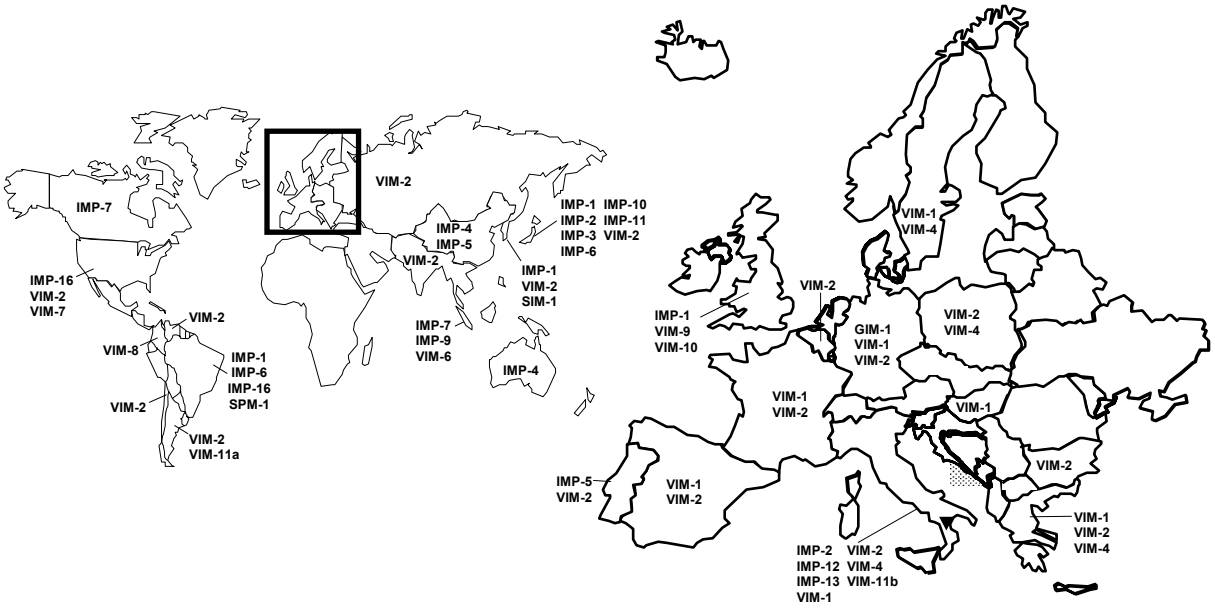

Figure 1.6 - Current spread of acquired metallo-β-lactamases (adapted from Bebrone, 2007).

A panoply of structurally different coumponds has been tested *in vitro* for their potential inhibition of metallo-β-lactamases. However, there are obstacles to the use of carbapenems combined with β-lactamase inhibitors: (1) metallo-β-lactamases present slight but important variations in their active site that difficult the design of a unique inhibitor; (2) contrasting with clavulanic acid, metallo-β-lactamases do not form stable intermediates; (3) there is a lack of studies about strains containing metallo-β-lactamase genes, in order to understand if the presence of an inhibitor has a decreased effect in MIC values; (4) metallo-β-lactamases have active site motifs similar to those for mammalian enzymes (e. g. human glyoxalase II), thus the use of inhibitors could represent a high level of toxicity to the patient (Walsh *et al.*, 2005).

3.3.9. Other β-lactamases

There are other unusual enzymes that also confer an ESBL phenotype and have been identified individually worldwide (e.g. BES-1, VEB-1, PER, SFO-1, GES, TLA-1) (Table 1.13). Enzymes like PER-1, PER-2, VEB-1, CME-1 and TLA-1 are related to each other, but present only 40 to 50% of homology and all of them confer resistance to oxyimino-cephalosporins (Bradford, 2001).

ESBL enzymes like PER-type share about 25 to 27% homology with TEM- and SHV-type enzymes (Paterson & Bonomo, 2005). First detected in *P. aeruginosas* in 1991, PER-1 efficiently hydrolyzes penicillins and cephalosporins and is susceptible to β-lactamase inhibitors. PER-2, which shares 86% homology with PER-1, has been found in Argentina in 1996. While PER-1 is reported mostly in Turkey and Korea, reports of PER-2 are so far restricted to South America (Naas *et al.*, 2008).

Another rare ESBL enzyme, the plamid-mediated VEB-1, which shares 38% homology with PER-1 and PER-2, confers high-level resistance to third generation cephalosporins and aztreonam, but is susceptible to clavulanic acid. VEB-1 β-lactamase was first reported in 1996 in an *E. coli* isolate from a Vietnamese patient. Other VEB enzymes have been identified in Kuwait and China (Paterson & Bonomo, 2005).

Table 1.13 - Plasmid-encoded extended-spectrum β-lactamases not belonging to the TEM, SHV, CTX-M and OXA families (adapted from Naas *et al.*, 2008)

β-Lactamase name	Year[a]	No. of variants	Origin of the name
'Minor ESBL'			
SFO-1	1988	1	*Serratia fonticola*
TLA-1	1991	1	Tlahuicas (Indian tribe)
PER	1991	3	Pseudomonas extended resistance
VEB	1996	5	Vietnam ESBLs
BES-1	1996	1	Brazilian ESBLs
GES	1998	9	Guyana ESBLs
BEL-1	2005	1	Belgium ESBLs
TLA-2	2005	1	51% amino-acid identity with TLA-1

[a] Year first recorded.

Enzymes of the GES type, also called IBC (integron-borne cephalosporinase), have been reported in widely separated countries (Poirel *et al.*, 2006). First described in France, GES-1 presents activity against penicillins and extended-spectrum cephalosporins, but is inhibited by β-lactamase inhibitors. This enzyme does not hydrolyse aztreonam, cephamycins and carbapenems. The GES-2 variant also hydrolyzes carbapenems and is

less inhibited by clavulanic acid, just like GES-4, GES-5 and GES-6. Several outbreaks of bacteria producing GES-enzymes have been reported in Korea, Greece, The Netherlands and South Africa (Naas *et al.*, 2008).

Others ESBL, like SFO, TLA, BES and BEL, are unique enzymes. SFO-1 was detected in 1988 in Japan and efficiently hydrolyses cefotaxime, but not ceftazidime, and is inhibited by clavulanic acid. The plasmid-mediated BES-1 enzyme was detected in 1996 in a patient from Brazil, and presents 48% amino acid identity with the CTX-M group 1 enzymes. It confers high-level resistance to cefotaxime but low-level resistance to ceftazidime (Bonnet *et al.*, 2000a). Identified in a *P. aeruginosa* strain, BEL-1 was reported in Belgium in 2004 and hydrolyses narrow- and extended-spectrum cephalosporins and aztreonam, but is inhibitied by clavulanic acid. The bla_{BEL-1} gene is associated with a class 1 integron located in the chromossome (Naas *et al.*, 2008). Reported in Mexico in 1993, TLA-1 hydrolyses extended-spectrum cephalosporins, aztreonam and cefepime, and is well inhibited by tazobactam and less by clavulanic acid. Sharing 51% amino acid identity with TLA-1, TLA-2, detected in 2002 in Germany, presents an increased catalytic efficiency against most cephalosporins but not against penicillins. Weak or no inhibition by β-lactamase inhibitors was detected *in vitro*, although MICs of cephalosporins were significantly lowered by their addition (Girlich *et al.*, 2005).

3.4. Detection of β-lactamases

The detection of β-lactamase production can be performed by different methods, such as chromogenic reactions or detection of the destruction of antibiotic activity. Nitrocefin, an example of a chromogenic method, is fast, more convenient and highly sensitive to most β-lactamases. Methods such as acidimetric and iodometric tests, in which the color changes are on a linked reaction, are cheaper than nitrocefin, but they are more prone to false-positive results (Livermore, 1995). However, chromogenic tests do not identify the type of β-lactamase present.

Other methods for detection of β-lactamase production, based on detection of the destruction of antibiotic activity, include methods like broth dilution and disk diffusion. Although these tests are slower than chromogenic methods, they present extreme sensitivity. With the constant emergence of new ESBLs or variations of already known ESBLs, the establishment of rapid and reliable laboratory methods for screening and confirmation of ESBL production is of great importance (Table 1.14).

Table 1.14 - Laboratory tests for detection of extended-spectrum β-lactamases (adapted from Samaha-Kfoury & Araj, 2003)

Tests	Method and interpretation
Screening tests	
Double disk approximation or double disk synergy	Disk of third generation cephalosporins placed at 30 mm distance from amoxicillin-clavulanic acid. Enhanced inhibition indicates ESBL
Microdilution test	Growth in a broth containing 1 µg/mL third generation cephalosporins indicates ESBL
Confirmatory tests	
MIC broth dilution	MIC of third generation cephalosporins alone or combined with clavulanic acid. A decrease in the MIC of the combination of ≥3 two-fold dilutions indicates ESBL
Combination disk	Uses two discs of third generation cephalosporins alone and combined with clavulanic acid. An increase in the zone inhibition of >5 mm with the combination disk indicates ESBL
Commercial tests	
Etest for ESBLs	Two sided strip containing ceftazidime on one side and ceftazidime-clavulanic acid on the other. The ratio of the MIC of the combination to that of ceftazidime alone of >8, or the presence of a phantom zone (or both) indicates ESBL
Vitek ESBL cards	Measures MICs and compares growth of bacteria in presence of cefotaxime and ceftazidime, alone, and in combination with clavulanic acid
MicroScan panels	Dehydrated panels for MIC evaluation by microdilution testing, which contain combinations of ceftazidime or cefotaxime plus β-lactamase inhibitors. An isolate is considered positive by the ESBL confirmation test if there is a ≥3 two-fold dilution drop in a MIC value the antibiotic tested alone as compared to the MIC value of the antibiotic tested with clavulanic acid.
BD Phoenix Automated Microbiology System	Shorth-incubation system that uses growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulanic acid
Molecular tests	
DNA probes, PCR, RFLP	Targets specific nucleotide sequences to detect different variants of differents β-lactamase family of genes

Studies comparing commercial methods with double disk tests showed that the highest sensitivity (99%) was obtained with BD Phoenix and the lower with MicroScan (84%). On the other hand, specificity was more variable, ranging from 52% (BD Phoenix) to 85% (Etest strips) (Wiegand *et al.*, 2007). However, manually performed double-disk tests presented the highest positive predictive value (98%) and one of the highest specificitie (97%) of all test methods.

Different levels of resistance against extended-spectrum cephalosporins can be detected among strains producing different ESBL enzymes. While some clearly confer high-

level resistance to these antibiotics, others can confer only intermediate-level resistance or even no resistance at all, to one or more third generation cephalosporins. Although a possible explanation for these differences could involve the inoculum effect (Katsanis *et al.*, 1994), routine clinical microbiology laboratories require ESBL detection methods and guidelines which are sensitive enough to recognize the level of resistance.

As such, ESBL-producing organisms may appear susceptible or resistant according to which breakpoints are used. With susceptibility defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) as 'a level of antimicrobial activity associated with a high likelihood of therapeutic success' (http://www.EUCAST.org), and resistance as 'a level of antimicrobial activity associated with a high likelihood of therapeutic failure', it is suprising that breakpoint differences are quite considerable between different countries (Table 1.15). The breakpoins of the Clinical and Laboratory Standards Institute (CLSI), formely National Committee for Clinical Laboratory Standards, are the most commonly used guidelines worldwide. However, other countries like France and the United Kingdom, present their own breakpoints, from the Comité de L'Antibiogramme de la Société Française de Microbiologie (SFM) and the British Society for Antimicrobial Chemotherapy (BSAC), respectively.

Table 1.15 - Comparision of MIC breakpoints for *Enterobacteriaceae* according to national guidelines (adapted from Paterson & Bonomo, 2005)

Country	MIC breakpoint[a] (µg/ml)			
	Cefotaxime		Ceftazidime	
	S(≤)	R (≥)	S(≤)	R (≥)
United States of America (CLSI)	8	64	8	32
United Kingdom (BSAC)	1	2	2	4
France (SFM)	4	32	4	32
The Netherlands (CRG)	4	16	4	16
Germany (DIN)	2	8	4	32
Norway (NWGA)	2	16	2	16
Sweden (SRGA)	4	32	4	16

[a] S, susceptible; R, resistant.

Studies performed in the United States and Europe reported that errors in the detection of ESBL-mediated resistance are frequently encountered with both automated and disk diffusion methods (Perez *et al.*, 2007). In Italy, some studies showed that only 42% of independent clinical microbiology laboratories correctly detected all of the ESBL-producing enterobacteria presented for analysis, while in the United States of America, only 32% of clinical microbiology laboratories reportedly perform tests to detect ESBL production by *Enterobacteriaceae* (Paterson & Bonomo, 2005; Perez *et al.*, 2007).

Broth dilution and disk diffusion methods can be used for screening of ESBL producers and the use of more than one of the tested antibiotics would improve the sensivity of ESBL detection. After screening, ESBL confirmatory testing should be based in the use of both ceftazidime and cefotaxime alone and in combination with clavulanic acid (CLSI, 2007).

In *Enterobacteriaceae* producing inducible AmpC chromosomal enzymes, ESBLs are more difficult to detect by CLSI guidelines, because AmpC enzymes may be induced by clavulanic acid, which may increase resistance to cephalosporins, overcoming the synergy arising from inhibition of the ESBL (Hanson, 2003). However, BSAC and SFM suggest the use of cefepime and cefpirome with clavulanic acid to compensate this problem (Perez *et al.*, 2007).

Other methods like IEF could be useful for narrowing down the possible identification of the ESBL enzyme. However, with the constant emergence of new ESBL and the growing number of enzymes sharing the same isoelectric points, such as it happens, for example, in the SHV, OXA and CTX-M families, discourages the exclusive use of this method. The most common molecular method of detecting and identifying the β-lactamases present, is PCR and sequencing with oligonucleotide primers specific for β-lactamase encoding genes. Several different PCR methods have been tested for different families of β-lactamase encoding genes, including restriction fragment length polymorphism of amplified products (PCR-RFLP), multiplex PCR, single strand conformation polymorphism of PCR products (PCR-SSCP), real-time PCR and microarrays (Bradford, 2001; Chia *et al.*, 2005; Randegger and Hächler, 2001b; Zhu *et al.*, 2007).

CHAPTER II

Occurrence of a novel SHV-type enzyme (SHV-55) among isolates of *Klebsiella pneumoniae* from Portuguese origin in a comparison study for extended-spectrum β-lactamase-producing evaluation

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ABSTRACT

Fifty-five isolates of *K. pneumoniae* were evaluated for ESBL detection and confirmation, using MIC testing by agar dilution, broth microdilution, and the ESBL Etest (AB biodisk, Solna, Sweden), according to reference laboratory criteria (RLC) and CLSI guidelines. The RLC classify as ESBL producers those strains for which any MIC of cephalosporins is 3-fold lower in the presence of 2 µg/mL of clavulanate. The Etest was the only to show 100% sensitivity and specificity to detect ESBL-producer strains with either set of guidelines. MIC determination by agar dilution or broth microdilution, using NCCLS guidelines showed sensitivity of 92.9%. Nucleotide sequencing allowed to the identification of a new ESBL (SHV-55). Overall, this gold standard method confirmed the production of 18 ESBL-producers, 36 non-ESBL-producers, from which nine were false ESBL producers (suggesting hyperproduction) and one a presumptive ESBL TEM-derived. New guidelines for ESBL detection and reliable methods of ESBL identification are required.

INTRODUCTION

Antimicrobial resistance based on hydrolysis of the antibiotic by β-lactamases is currently a worldwide problem (Bradford, 2001). The overuse of new extended-spectrum cephalosporins and monobactams has allowed to the emergence of new resistant *K. pneumoniae* strains, some producing ESBL (http://www.lahey.org/studies). These ESBLs are variants of parental enzymes, carrying one or more amino acid substitutions that modify the substrate specificity of the enzyme.

Resistance due to ESBL production can be difficult to detect because *K. pneumoniae* can hyperproduce non-ESBL β-lactamases (e.g., SHV-1) or possess modifications in outer membrane proteins, which also confer an ESBL similar phenotype causing false positive results (Rice *et al.*, 2000; Schwaber *et al.*, 2004; Wu *et al.*, 2001). Inhibitor-resistant TEM enzymes and AmpC-type β-lactamase coexisting with ESBL enzymes can also compromise the inhibition of the ESBL enzyme by clavulanate, as the synergistic effect of the β-lactamase inhibitor and cephalosporins against ESBL is more difficult to visualize or is not detectable (Tzouvelekis *et al.*, 1999). Indeed, inhibition by β-lactamase inhibitors, such as clavulanate or sulbactam, is the common property of ESBL enzymes and is used for the phenotypic confirmation of ESBL production by an isolate. There are various methods for confirming ESBL production. Evaluations of these methods have been published (Florijn *et al.*, 2002; Komatsu *et al.*, 2003; Leverstein-van Hall *et al.*, 2002; Linscott & Brown 2005, MacKenzie *et al.*, 2002).

Here, we compared phenotypic methods and guidelines used for the confirmation of clinical *K. pneumoniae* ESBL-producing strains of Portuguese origin. Our RLC was compared with the CLSI guidelines for interpreting confirmatory tests. Nucleotide sequencing was used as the gold standard method to distinguish between ESBL and non-ESBL isolates and to identify which ESBL type is present.

MATERIALS AND METHODS

Setting and bacterial isolates. Fifty-five *K. pneumoniae* strains isolated from clinical specimens were randomly selected from the strain collection of the Antibiotic Resistance Unit at the National Institute of Health (NIH) in Lisbon, ensuring that all isolates were from different patients. Each isolate was identified with the API 20E System (bioMérieux, Marcy l'Étoile, France). Control strains used in antimicrobial susceptibility determination were the non-ESBL-producing *E. coli* ATCC 25922, *K. pneumoniae* INSRA558, and the ESBL-producer *E. coli* INSRA1248 (TEM-24). Control strains used for IEF of SHV-55 producers were *E. coli* INSRA99 (IRT-2, pI 5.2), *E. coli* RP4 (TEM-2, pI 5.6), *K. pneumoniae* INSRA558 (SHV-28, pI 7.6), and *E. coli* SolRI 90 (AmpC, pI 9.2). *E. coli* R111 (TEM-1, pI 5.4; bla_{TEM-1} plus *ampC*), *S. enterica* serovar Typhimurium (OXA-1, pI 7.4; bla_{OXA-1}), *E. coli* C600 (SHV-1, pI 7.6; bla_{SHV-1}) and *E. coli* UA1526 (CTX-M-15, pI 8.9; $bla_{CTX-M-15}$) were used as control strains for both PCR and IEF.

Antibiotic susceptibility determination and ESBLs detection. The MIC of various antibiotics was each determined by an agar dilution method, according to the SFM, which was considered as the reference method (Cavallo *et al.*, 2005). The results obtained were interpreted using the same guidelines, except for cefoperazone for which the NCCLS (2004) guidelines were used. Our criteria – the RLC – classify as ESBL producers those strains for which any MIC of cephalosporins is three-fold lower in the presence of 2 µg/mL of clavulanate.

Phenotypic confirmatory tests for ESBLs. ESBL production was confirmed by three phenotypic tests: 1) the Etest ESBL strips (AB Biodisk, Solna, Sweden), with ceftazidime, ceftazidime plus clavulanate, cefotaxime, and cefotaxime plus clavulanate; 2) MIC determination by a broth microdilution method (MicroScan ESBL Plus Confirmation Panel, Dade Behring, West Sacramento, CA) for ceftazidime, ceftazidime plus clavulanate, cefotaxime, and cefotaxime plus clavulanate; and 3) MIC determination by agar dilution method. Results from the three confirmatory methods were interpreted according to CLSI guidelines: an MIC value of at least one extended-spectrum cephalosporin (ceftazidime

and/or cefotaxime) was ≥2 µg/mL and was decreased by ≥ 3- to two-fold serial dilution by the presence of clavulanate. Tests 1 and 2 were also interpreted according to the RLC. The results of the different tests for ceftazidime, ceftazidime plus clavulanate, cefotaxime and cefotaxime plus clavulanate were compared.

IEF. IEF tests of cell extracts from isolates producing SHV-55 enzymes were performed as previously described (Caniça *et al.*, 1997a).

Conjugation. Liquid mating experiments were conducted with streptomycin-, nalidixic acid- and rifampicin-resistant recipient strains of *E. coli* C600 to determine if the ESBL phenotype of the SHV-55 enzyme was transferable. Mueller-Hinton containing 1 mg of cefotaxime per liter and 300 mg of rifampicin or 50 mg of nalidixic acid or 20 mg of streptomycin per liter was used for selecting transconjugants.

PCR and Sequencing. Total DNA was prepared as previously described (Féria *et al.*, 2002). The genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$ and *ampC* were detected by PCR at DNA Engine Dyad^{TM} (MJResearch, Waltham, MA, USA) in 25 µl reaction volume containing 0.5 mmol/L of each of the 4 deoxynucleotides (Roche Diagnostics GmbH, Mannhein, Germany), 0.2 µmol/L of each primer (Table 2.1), 3.0 mmol/L $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Qiagen, Izasa, Lisbon, Portugal), 1x Q-Solution (Qiagen) and 1x buffer (Qiagen). PCR conditions were: 7 minutes at 94ºC, followed by 30 cycles of 30 s at 94ºC, 1 minute at 56ºC (for *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes) or 61ºC (for *bla*$_{OXA}$ and *ampC* genes) and 30 s at 72ºC, and concluded by 10 minutes at 72ºC. PCR products were purified with ExoSAP IT (USB Corporation, Cleveland, OH), and all amplicons from phenotype-like-ESBL- and phenotypic-like non-ESBL-producer strains were further sequenced directly on both strands using automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK). Sequencing was used as the gold standard method for identifying enzymes according to the gene sequences.

Table 2.1 - Primers used for PCR amplification and sequencing

β-lactamase gene	Primer Name	Primer Sequence	Position (5'→3')	Reference
bla$_{TEM}$	FIN	5'-ATTCTTGAAGACGAAAGGGC-3'	0/19	Belaaouaj *et al.*, 1994
	DEB	5'-ATGAGTAAACTTGGTCTGAC-3'	1091/1072	Caniça *et al.*, 1997b
bla$_{SHV}$	SHVsf	5'-CGCTTCTTTACTCGCCTTTA-3'	-50/-31	This study
	SHVsr	5'-TTACGCTTGCCAGTGCTC-3'	861/844	
bla$_{OXA}$	oxa1f	5'-TATCTACAGCAGCGCCAGTG-3'	929/948	Féria *et al.*, 2002
	oxa1r	5'-CGCATCAAATGCCATAAGTG-3'	1127/1108	
ampC	ampCf	5'-CCCCGCTTATAGAGCAACAA-3'	157/176	Féria *et al.*, 2002
	ampCr	5'-TCAATGGTCGACTTCACACC-3'	791/772	
bla$_{CTX-M}$	CTXf	5'-TTTGCGATGTGCAGTACCAGTAA-3'	205/227	Edelstein *et al.*, 2003
	CTXr	5'-CGATATCGTTGGTGGTG CCATA-3'	748/727	

Statistic analysis. To evaluate ESBL detection and confirmatory methods, we calculated the sensitivity, specificity, and positive and negative predictive values for all strains using SFM method and RLC interpretative criteria. This method and criteria was considered as reference for comparisons. We used a 2-way table for calculation of efficiency values.

Nucleotide sequence accession number. The *bla*$_{SHV-55}$ nucleotide sequence was submitted to the EMBL Nucleotide Sequence Database under accession number AJ863560.

RESULTS

Evaluation of screening and phenotypic confirmatory tests for ESBLs. Among the 55 isolates of *K. pneumoniae*, the reference method and criteria detected 28 strains as putative ESBL producers (Table 2.2) and 27 as non-ESBL producers (data no shown). Three phenotypic methods (MIC by agar dilution, MIC by broth microdilution and Etest ESBL strips) with both RLC and CLSI interpretations were used to confirm ESBL (Table 2.2).

Using the CLSI guidelines to interpret the results we detected 92.9% of the phenotype-like ESBL-producing strains (Table 2.3). Broth microdilution and RLC interpretation identified a putative ESBL producer (strain 3330, data not shown) with MIC for ceftazidime of 1 µg/mL, for ceftazidime plus clavulanate of ≤ 0.12/4 µg/mL, for cefotaxime of ≤ 0.5 µg/mL and for cefotaxime plus clavulanate of ≤ 0.12/4 µg/mL (specificity 96.2%). This strain was classified as a non-ESBL by all other tests including sequencing. ESBL detection by agar dilution or broth microdilution interpreted by CLSI guidelines gave three false negative ESBL producers (strains 3028, 3805 and 3807), all of which contained the ESBL enzyme SHV-2A (Table 2.2).

The agar dilution method identified four strains (strains 251A, 1228, 1865A and 2613) with synergy only between ceftazidime and clavulanate and two strains (strains 3805 and 3807) with synergy only between cefotaxime and clavulanate. The Etest detected all ESBL producers with either RLC or CLSI guidelines (sensitivity 100% and specificity 100%) (Table 2.3). Broth microdilution and the Etest confirmed 27 strains as non-ESBL, as did the reference method.

Values set in boldface shows CIM values by RLC with MIC decrease of ≥3 doubling dilutions between CAZ or CTX alone and in combination with clavulanate. AD = agar dilution method; BMD = broth microdilution method; AMX = amoxicillin; AMC = amoxicillin with clavulanate; CAZ = ceftazidime; CAZ/CLA = ceftazidime with clavulanate; CTX = cefotaxime; CTX/CLA = cefotaxime with clavulanate.
[a] Also used as initial screen test for ESBL.
[b] Identification by nucleotide sequencing.
[c] Two micrograms of clavulanic acid per millilitre.
[e] More than 1 *bla*$_{SHV-1}$ genes with different frameworks present.
[f] More than 1 *bla*$_{TEM}$ genes present. Putative ESBL TEM derived.

Table 2.2 - Comparison of phenotypic methods and interpretative criteria for ESBL in 28 putative producer strains of *K. pneumoniae*, and enzymes deduced from the nucleotide sequences of the genes

Strain no.	MIC (µg/ml) by AD under RLC [a]						ESBL confirmation under RLC [a]		ESBL confirmation under CLSI			Enzymes [b]
	AMX	AMC [c]	CAZ	CAZ/CLA [c]	CTX	CTX/CLA [c]	BMD	Etest	AD	BMD	Etest	
251[a]	>4,096	64	8	0.25	0.06	0.06	+	+	+	+	+	TEM-1[a]/SHV-1
371	>4,096	128	32	2	4	0.125	+	+	+	+	+	SHV-1[d]
604	>4,096	64	256	0.5	0.5	≤0.016	+	+	+	+	+	TEM-10/SHV-1/SHV-11
640	>4,096	256	32	1	4	0.125	+	+	+	+	+	SHV-1[d]
668	>4,096	128	256	0.5	1	0.03	+	+	+	+	+	TEM-10/SHV-1[d]
674	>4,096	256	64	4	4	0.06	+	+	+	+	+	SHV-1[d]
1001	>4,096	64	>256	0.5	1	0.06	+	+	+	+	+	TEM-10/SHV-28
1030	>4,096	64	32	0.06	0.5	0.06	+	+	+	+	+	TEM-1/TEM-10/SHV-28
1205	>4,096	512	32	0.5	4	0.125	+	+	+	+	+	SHV-1[d]
1228	>4,096	512	2	0.125	0.125	0.06	+	+	+	+	+	SHV-1
1242[a]	>4,096	512	64	2	4	0.125	+	+	+	+	+	SHV-1[d]
1302	>4,096	512	128	2	4	0.125	+	+	+	+	+	SHV-1[d]
1865[a]	4,096	16	2	0.125	0.03	≤0.016	+	+	+	+	+	TEM-1[a]/SHV-1
2613	>4,096	256	2	0.25	0.06	0.03	+	+	+	+	+	TEM[e]/SHV-28
2638	2,048	8	32	0.25	0.25	0.03	+	+	+	+	+	TEM-24/SHV-11
2695	>4,096	32	>256	2	4	0.125	+	+	+	+	+	TEM-24/SHV-1[d]
2934	4,096	4	64	1	4	0.06	+	+	+	+	+	SHV-55
3028	>4,096	16	1	0.125	2	0.06	+	+	+	-	+	TEM-1B/SHV-2[a]/ SHV-11
3054	>4,096	16	1	0.125	2	0.06	+	+	+	+	+	TEM-1B/SHV-2A
3607	4,096	8	256	0.5	8	0.06	+	+	+	+	+	SHV-5/ SHV-12
3775	>4,096	256	64	0.125	4	0.03	+	+	+	+	+	TEM-1B/SHV-55
3776	>4,096	8	64	0.125	4	0.03	+	+	+	+	+	SHV-55
3805	>4,096	8	1	0.25	1	0.03	+	+	-	+	+	TEM-1B/SHV-2A
3807	>4,096	4	0.125	0.06	0.5	0.03	-	+	-	-	+	TEM-1B/SHV-2A
3812	>4,096	≤2	64	0.06	4	≤0.016	+	+	+	+	+	TEM-3/SHV-1
3955C	>4,096	≤2	128	0.25	4	0.03	+	+	+	+	+	SHV-55
3972	>4,096	256	64	0.5	4	0.03	+	+	+	+	+	TEM-1B/SHV-55
4221	>4,096	16	64	0.5	2	≤0.016	+	+	+	+	+	TEM-10/SHV-11

Table 2.3 - Comparison of the sensitivities, specificities, positive predictive value (PPV) and negative predictive value (NPV) of three methods, interpreted by CLSI guidelines or RLC, used as confirmatory test for 55 phenotype-like ESBL and non-ESBL *K. pneumoniae*[a]

Test/Interpretation criteria	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Broth Microdilution				
RLC	96.4	96.2	96.4	96.2
CLSI	92.9	100	100	93.1
Etest ESBL				
RLC	100	100	100	100
CLSI	100	100	100	100
Agar dilution				
CLSI	92.9	100	100	93.1

[a] Reference method was agar dilution interpreted by RLC, used for ESBL detection and confirmation.

Identification of genes coding for ESBLs. PCR detected the presence of genes coding for β-lactamases of the SHV and TEM families, but did not discriminate between different variants of TEM or SHV. No bla_{OXA}, bla_{CTX-M} or *ampC* gene was detected in our strains. However, sequencing was unable to identify one putative TEM-derived (strain 2613) enzyme, possibly responsible for an ESBL phenotype, due to the presence of more than one sequence of the bla_{TEM} gene detected by gold standard method. We detected nine false ESBL producers (strains 251A, 371, 640, 674, 1205, 1228, 1242A, 1302 and 1865A), six of which presented more than one overlapping bla_{SHV-1} sequence. Sequencing of all 27 non-ESBL isolates revealed parental bla_{SHV} and bla_{TEM} genes (data not shown).

Sequencing also identified a bla_{SHV} coding for the new ESBL enzyme, named SHV-55 (Table 2.2). This enzyme presented three substitutions in the aminoacid sequence: Tyr7 (TAT)→Phe (TTT), Gly238 (GGC) →Ser (AGC) and Glu240 (GAG) →Lys (AAG). The new bla_{SHV-55} gene was not self-transferable to *E. coli* receptor strains. Strains 3775 and 3972 produced SHV-55 plus TEM-1 enzymes, which could be responsible for the high MICs of amoxicillin plus clavulanate, piperacillin, piperacillin plus tazobactam, and mecillinam (Table 2.4). The MICs of ceftazidime for SHV-55 producers were 64 to 128 µg/mL, of cefotaxime 4 µg/mL, of aztreonam 128 to 256 µg/mL and of ceftriaxone 4 to 16 µg/mL. The presence of clavulanate decreased the MIC of third-generation cephalosporins or monobactams by between 6- and 11-fold doubling dilutions (Table 2.4).

Table 2.4 - MIC values for 5 clinical strains producing the new SHV-55 enzyme [a] and strains harboring SHV-28 or SHV-5

Antimicrobial Agent	MICs (µg/mL) for the following Kp and Ec strains (with the indicated enzyme):							
	Kp 2934 (SHV-55)	Kp 3775 (SHV-55 + TEM-1)	Kp 3776 (SHV-55)	Kp 3955C (SHV-55)	Kp 3972 (SHV-55 + TEM-1)	INSRA Kp558 (SHV-28)	Ec BM694[b] (SHV-5)	Kp 956[c] (SHV-5)
AMX	4,096	>4,096	>4,096	>4,096	>4,096	64	>2,048	ND
AMC	4	256	8	≤2	256	4	ND	8
TIC	>4,096	>4,096	>4,096	>4,096	>4,096	128	>1,024	ND
PIP	128	>512	128	128	>512	4	128	>512
TZP	8	256	4	4	128	2	ND	ND
MEC	1	32	2	2	16	0.25	ND	ND
CEF	128	256	128	256	256	4	256	512
CXM	16	16	16	16	16	4	ND	32
CFP	16	32	8	8	32	0.5	ND	ND
ATM	256	128	128	128	128	0.125	256	128
ATM/CLA	0.125	0.125	0.125	0.125	0.25	0.125	ND	ND
CAZ	64	64	64	128	64	0.125	128	64
CAZ/CLA	1	0.125	0.125	0.25	0.5	0.125	ND	ND
CRO	16	4	4	4	8	0.03	8	8
CRO/CLA	0.125	0.06	0.06	≤0.016	0.125	0.03	ND	ND
CTX	4	4	4	4	4	0.03	16	8
CTX/CLA	0.06	0.03	0.03	0.03	0.03	0.03	ND	ND
FEP	1	0.5	0.5	0.5	1	0.25	ND	ND
FOX	4	4	8	8	4	8	32	4
IPM	0.25	0.125	0.25	0.25	0.25	0.25	0.125	0.25

Kp = *K. pneumoniae*; Ec = *E. coli*; ND = not determined; TIC = ticarcillin; PIP = piperacillin; TZP = piperacillin with tazobactam; MEC = mecillinam; CEF = cephalothin; CXM = cefuroxime; CFP = cefoperazone; ATM = aztreonam; ATM/CLA = aztreonam with clavulanate; CRO = ceftriaxone; CRO/CLA = ceftriaxone with clavulanate; FEP = cefepime; FOX = cefoxitin; IPM = imipenem.

[a] Agar dilution according to SFM guidelines (Cavallo *et al.*, 2005).

[b] From Gutmann *et al.*, 1989.

[c] From Mulgrave and Attwood, 1993.

DISCUSSION

The emergence and rapid dissemination of ESBL-producing strains of *K. pneumoniae* are responsible for outbreaks of infection worldwide. Intensive care units are particularly sensitive in terms of clinical outcomes, and these organisms have been implicated in increased morbidity and mortality (Paterson & Bonomo, 2005). There are difficulties with the routine laboratory detection of ESBL which can be complex and misleading, and this restricts treatment options (Bradford, 2001; Paterson & Bonomo, 2005).

The phenotypic methods gave nine false-positive classifications as ESBL producers, and the reasons include: modifications in outer membrane proteins (Rice *et al.*, 2000), and hyperprodution of SHV-1 enzyme due to high gene copy number (Podbielski *et al.*, 1991a), or a single base pair change in promoter sequence (Jacoby & Han, 1996; Rice *et al.*, 2000). This can compensate for the low specific activity of the β-lactamase, allowing such isolates to cause diseases. Furthermore, it has been reported that a larger inoculum *in vivo* increases the success of infections of such strains (Jacoby & Han, 1996). Alternatively, these nine false ESBL producers may produce enzymes other than those studied, although the specific PCR for $bla_{\text{CTX-M}}$ and the MIC values for cefepime exclude enzymes of CTX-M family, because these enzymes efficiently hydrolyze cefepime (Sabaté *et al.*, 2002). The reference method identified a false-positive ESBL producer (strain 3330), possibly due to hyperproduction of $bla_{\text{SHV-1}}$ or outer membrane proteins changes.

We found that the broth microdilution method with CLSI guidelines or RLC was less sensitive than the Etest with either criteria or agar dilution using RLC. The Etest was the only method, other than the reference method, to detect all the ESBL producers. However, it has some limitations, including reading and interpretation problems (Leverstein-van Hall *et al.*, 2002). Nevertheless, with either set of interpretative criteria, we and other obtained 100% sensitivity with the Etest (Leverstein-van Hall *et al.*, 2002). Some authors have reported a lower sensitivity: Florijn *et al.* (2002), 93%; Linscott & Brown (2005), 97%; and MacKenzie *et al.* (2002), 77%. The broth microdilution method gave a sensitivity of 93% using CLSI guidelines, to be compared with results in other studies: Komatsu et al. (2003), 92%; Linscott & Brown (2005), 100%.

Some of the ESBL producers had MIC values in the susceptible range according to CLSI criteria, suggesting that the synergy between the antibiotic and the inhibitor should be pointed out. Based only on the phenotype and with concentrations of ≥ 2 μg/mL, CLSI detection and confirmation methods may give false-positive and false-negative results for isolates with uncommon β-lactamases (Schwaber *et al.*, 2004), besides SHV, TEM or CTX-M.

However, increasing the breakpoint to ≥ 4 µg/mL for ceftazidime, as suggested by Moland *et al.* (1998), would decrease the apparent number of ESBL-like-phenotype isolates in our study by 17.9%, including two true positives with SHV-2A. Our study indicates that a screening test by agar dilution considering the susceptibility range under MIC of 2 µg/mL and synergy with clavulanate, or the Etest method, can be very reliable. The optimal laboratory method for ESBL detection has not yet been identified. Phenotypic methods are more reliable and less costly than genotypic methods for routine laboratory testing.

However, the importance of genotypic methods is reflected here by sequencing all strains, which allowed us to identify a new ESBL encoding gene among other gene β-lactamases. Thus, we report a new ESBL enzyme, SHV-55, that carries the variations of both SHV-5 (Gly238Ser and Glu240Lys) and SHV-28 (Tyr7Phe, in the signal peptide region) β-lactamases (http://www.lahey.org/studies/webt.htm). SHV-5 (with pI 8.2) was initially detected in a *K. pneumoniae* isolate from a patient attending a hospital in Santiago, Chile (Gutmann *et al.*, 1989), and was subsequently widely found in other countries (Mulgrave & Attwood, 1993). Non-ESBL enzyme, SHV-28 (with pI 7.6), was first detected in a *K. pneumoniae* isolate in China in the year 2000 (accession number AF299299, unpublished) and again in China by another team in the year 2002 (accession number AF538324, unpublished). SHV-28, unlike ESBL, was not associated with the hydrolysis of broad-spectrum cephalosporins or monobactams (Table 2.4). The positive charge on amino-terminal region of the signal peptide plays an important role in protein secretion across the membrane (Inouye *et al.*, 1982); however, the neutral charge resulting from the Tyr7Phe substitution appears not to have any functional significance for the expression of the resistance phenotype. SHV-55, like SHV-5, confers greater resistance to ceftazidime and aztreonam than to cefotaxime (Gutmann *et al.*, 1989; Mulgrave & Attwood 1993). The coexpression of SHV-55 and TEM-1 enzymes in two strains (3775 and 3972) presumably explains their greater resistance to amoxicillin plus clavulanate, piperacillin, piperacillin plus tazobactam and mecillinam.

Our study shows that the detection and confirmation of ESBL-producing strains is a problem and that *K. pneumoniae* producing ESBL enzymes are probably overlooked in some hospitals and private laboratories. This may constitute a real threat because 1) there is an inoculum effect *in vivo*, as well as *in vitro*, with a consequent rise in resistance to expanded-spectrum cephalosporins; 2) *K. pneumoniae* is a major nosocomial pathogen; and 3) it is a frequent ESBL producer worldwide. Improved guidelines for ESBL testing with more appropriate cephalosporin breakpoints and improved automated methods for the detection of this β-lactamases are needed.

CHAPTER III

Biochemical characterization of SHV-55, an extended-spectrum class A β-lactamase from *Klebsiella pneumoniae*

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TEXT

We biochemically characterized the *K. pneumoniae* extended-spectrum SHV-55 enzyme carrying the aminoacid substitutions Tyr7Phe (as in SHV-28), Gly238Ser and Glu240Lys (both found in SHV-5) identified in a previous study (Mendonça *et al.*, 2006a). SHV-55 ESBL differed from SHV-5 only in the signal peptide region (Gutmann et al., 1989). The bla_{SHV-55} gene was obtained as described by Mendonça et al (Mendonça et al., 2008), and transformants were selected on Luria Broth agar supplemented with 30 µg of kanamycin/ml and 16 µg of amoxicillin/ml. SHV-55 was extracted and purified according to the previously described protocol (Mendonça *et al.*, 2008). The Michaelis constant (K_m) and catalytic activity (k_{cat}) of purified extracts of SHV-55 were obtained by using a computerized microacidimetric method and 702 SM Titrino pH-stat apparatus (Metrohm, Herisau, Swiss) (Labia *et al.*, 1973). The complete hydrolysis time-courses were analyzed, and the kinetic progress curves were fitted by nonlinear least-squares regression. These kinetic parameters were determined and compared to those of SHV-1 enzyme for 10 β-lactams (Table 3.1).

SHV-55 has a high affinity (K_m, 5 to 10 µM) for penicillins, similar to that of SHV-5 (Gutmann *et al.*, 1989), and higher than that of SHV-1 (K_m, 11 to 31 µM). SHV-55 presented higher affinity values (K_m, 9 to 58 µM) than SHV-1 (K_m, 40 to 257 µM) for narrow-, extended-, and broad-spectrum cephalosporins and monobactams. This finding may be a consequence of the Gly238Ser substitution present in the active site of both SHV-55 and SHV-5, which pushes the β-strand out and away from the reactive Ser70 (Huletsky *et al.*, 1993). This results in a slightly expanded active site that may improve binding and accommodate cephalosporins with bulky side-chains (Matagne *et al*., 1998).

SHV-55 presented a higher affinity for cefotaxime than for ceftazidime (K_m, 21 and 58 µM, respectively), as did SHV-5 (Gutmann *et al.*, 1989; Mulgrave & Attwood, 1993). This finding is surprising because both enzymes possesses the Glu240Lys substitution, which increases hydrolytic activity against ceftazidime due to the change in the electrostatic charge of the exposed group at position 240 (Huletsky *et al.*, 1993; Randegger *et al*., 2000). The enzymatic activity (k_{cat}s) of SHV-55 for penicillin G and amoxicillin were 84- and 45-fold lower, respectively, than those of SHV-1, and the catalytic efficiency (k_{cat}/K_m ratio) against penicillins was more than 10-fold higher for SHV-1 (k_{cat}/K_m ratio, 20 to 84 $\mu M^{-1}.s^{-1}$) than for SHV-55 (k_{cat}/K_m ratio, 2 to 5 $\mu M^{-1}.s^{-1}$). However, the enzyme activity and catalytic efficiency against extended- and broad-spectrum cephalosporins were higher for SHV-55 (k_{cat}, 7 to 24 s^{-1} and k_{cat}/K_m ratio, 0.2 to 1 $\mu M^{-1}.s^{-1}$) than for SHV-1 (note, however, that the values for monobactam were undeterminable), although the catalytic efficiency of the two enzymes against cephalothin were similar (k_{cat}/K_m ratio, 3.2 and 4.4 $\mu M^{-1}.s^{-1}$). This result may be due to the amino acid substitutions in SHV-55 causing conformational modifications in the active

site. Fifty percent inhibitory concentrations (IC_{50}s) indicated that SHV-55 was nine-fold more susceptible to the inhibitor activity of clavulanate than SHV-1 (IC_{50}s of clavulanate, 0.02 *versus* 0.17 μM).

Table 3.1 - Kinetic constants of SHV-55 and SHV-1 β-lactamases[a]

Antibiotic	SHV-1[b]				SHV-55			
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	IC_{50} (μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	IC_{50} (μM)
Clavulanic Acid				0.17				0.02
Penicillin G	23 ± 0.42	1,937 ± 82	84.3 ± 2.0		5 ± 0.51	23 ± 0.76	5.3 ± 0.42	
Amoxicillin	31 ± 1.29	1,044 ± 10	33.3 ± 1.1		10 ± 0.14	23 ± 0.17	2.5 ± 0.02	
Ticarcillin	11 ± 3.40	220 ± 49	20.4 ± 1.7		6 ± 0.02	8 ± 0.00	1.5 ± 0.00	
Piperacillin	24 ± 0.53	1,490 ± 96	62.5 ± 2.7		8 ± 0.37	27 ± 1.53	3.7 ± 0.03	
Cephalothin	40 ± 1.46	128 ± 33	3.2 ± 0.8		9 ± 0.68	38 ± 3.94	4.4 ± 0.78	
Cefuroxime	80 ± 0.59	<0.1	ND[c]		23 ± 0.53	7 ± 0.19	0.3 ± 0.00	
Ceftazidime	142 ± 3.18	<0.1	ND		58 ± 7.40	9 ± 0.21	0.2 ± 0.02	
Cefotaxime	257 ± 20.65	<0.1	ND		21 ± 0.13	24 ± 0.34	1.1 ± 0.01	
Aztreoname	ND	<0.1	ND		5 ± 0.62	<0.1	ND	
Cefepime	149 ± 7.30	<0.1	ND		149 ± 2.61	30 ± 3.10	0.2 ± 0.02	

[a] Values (except IC_{50}s) are means ± standard deviations.
[b] Data are from reference Mendonça *et al.* (Mendonça *et al.*, 2008).
[c] ND, Not determinable.

In conclusion, these results confirmed the extended-spectrum activity of SHV-55 enzyme, which is important due to the magnitude of extended- and broad-spectrum SHV β-lactamases described to date and not biochemically characterized, in spite to the ease of sequencing genes (http://www.lahey.org/studies).

CHAPTER IV

The Lys234Arg substitution in the enzyme SHV-72 is a determinant for resistance to clavulanic acid inhibition

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ABSTRACT

The new β-lactamase SHV-72 was isolated from clinical *K. pneumoniae* INSRA1229, which exhibited the unusual association of resistance to amoxicillin-clavulanic acid combination (MIC, 64 μg/ml) and susceptibility to cephalosporins, aztreonam, and imipenem. SHV-72 (pI 7.6) harbored the three amino acid substitutions Ile8Phe, Ala146Val and Lys234Arg. SHV-72 had high catalytic efficiency against penicillins (k_{cat}/K_m, 35 to 287 $\mu M^{-1}.s^{-1}$) and no activity against oxyimino β-lactams. The concentration of clavulanic acid necessary to inhibit the enzyme by 50% was 10-fold higher for SHV-72 than for SHV-1. Molecular dynamics simulation suggested that the Lys234Arg substitution in SHV-72 stabilized an atypical conformation of Ser130 side chain, which moved the Oγ atom of Ser130 around 3.5 Å away from the key Oγ atom of the reactive serine (Ser70). This movement may therefore decrease the susceptibility to clavulanic acid by preventing cross-linking between Ser130 and Ser70.

INTRODUCTION

The most common resistance mechanism in bacteria against β-lactam antibiotics is the production of β-lactamases (EC 3.5.2.6), which hydrolyze and inactivate β-lactams. β-Lactamases are divided into four major classes (A to D) on the basis of their primary sequence (Ambler, 1980). While class B is composed of metalloenzymes that necessitate the presence of zinc cations for activity, classes A, C and D are serine hydrolases (Ambler, 1980; Bush *et al.*, 1995). Class A enzymes comprise several enzyme families, including the clinically relevant enzymes TEM and SHV (Livermore, 1995).

TEM and SHV enzymes initially had preferential activity against penicillins, as in the case of enzymes SHV-1 and TEM-1. Oxyimino β-lactams that are resistant to their hydrolytic activity and β-lactam inhibitors, such as clavulanic acid and tazobactam, have been developed to get around the activities of these enzymes (Bradford, 2001; Chaïbi *et al.*, 1999; Livermore, 1995). Nevertheless, the presence of point mutations in TEM and SHV enzymes has expanded the substrate spectrum to include oxyimino β-lactams and/or has conferred resistance to the inhibitors (Babic *et al.*, 2006; Bradford, 2001; Chaïbi *et al.*, 1999; Livermore, 1995).

More than 28 inhibitor-resistant TEM enzymes have been detected. They harbor amino acid substitutions at positions 69, 130, 165, 182, 244, 275 and/or 276 that confer resistance to inhibitors (Chaïbi *et al.*, 1999; Helfand *et al.*, 2003). Only three natural IRSs have been reported (http://www.lahey.org/studies). It has been proposed that substitutions at positions 69, 130 and 187 are involved in their resistance to inhibitors (Chang *et al.*, 2001;

Dubois *et al.*, 2004, Prinarakis *et al.*, 1997). IRS enzymes have also been constructed *in vitro* by site saturation mutagenesis in position 244 (Thomson *et al.*, 2006).

In this study, we performed a phenotypic, molecular and biochemical characterization of the new IRS-type β-lactamase SHV-72 from a clinical *K. pneumoniae* strain and investigated by molecular dynamic simulations (MDSs) the role of the Lys234Arg substitution in its resistance to clavulanic acid.

MATERIALS AND METHODS

Bacterial strains and plasmid. *K. pneumoniae* INSRA1229 was isolated from sputum of an 80 year old male in an internal medicine service of a general hospital in Lisbon, in 1999. *E. coli* DH5α Δ*ampC* and plasmid pBK-CMV (Stratagene, Amsterdam, The Netherlands) were used for the cloning experiments (Table 4.1) (Delmas *et al.*, 2006). *bla*$_{SHV-1}$-encoding *E. coli* C600 was used as control strain for PCR.

Table 4.1 - Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
K. pneumoniae	Clinical strain harbouring the natural plasmid pINSRA1229	This study
E. coli C600	Strain harbouring the natural plasmid pINSRASHV-1	This study
E. coli DH5α	*sup*E44 Δ*lac*U169 (φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 Δ*ampC*	Delmas *et al.*, 2006
Plasmid		
pINSRASHV-1	Plasmid from *E. coli* C600 containing *bla*$_{SHV-1}$ gene; resistance phenotype: amoxicillin	This study
pINSRA1229	Natural plasmid from *K. pneumoniae* INSRA1229 containing *bla*$_{SHV-72}$ gene; resistance phenotype: IRS	This study
pBK-SHV-72	Recombinant plasmid containing 891-bp fragment with *bla*$_{SHV-72}$ gene; resistance phenotype: IRS, kanamycin	This study
pBK-SHV-1	Recombinant plasmid containing 891-bp fragment with *bla*$_{SHV-1}$ gene; resistance phenotype: amoxicillin, kanamycin	This study
pBK-CMV	Phagemid vector; resistance phenotype: kanamycin	Stratagene

Susceptibility testing. MICs were determined by agar dilution method, according to the recommendations of the SFM (Cavallo *et al.*, 2007). Strains were tested against cefotaxime (Sanofi Aventis), ceftriaxone and trimethoprim (Roche Pharmaceuticals), aztreonam and cefepime (Bristol-Myers Squibb), amoxicillin, cefuroxime, ceftazidime, clavulanic acid and ticarcillin (GlaxoSmithKline), cefoxitin (Labesfal), mecillinam (Leo Pharma), cephalothin (Sigma), cefoperazone (Pfizer), piperacillin and tazobactam (Wyeth Pharmaceuticals), imipenem (Merck Sharp & Dohme, Lda), ciprofloxacin (Bayer HealthCare), gentamicin

(Schering-Plough) and penicillin (Laboratórios Atral, S.A.). MICs of β-lactam antibiotics were determined alone and combined at a fixed concentration of clavulanic acid (2 µg/ml) (amoxicillin, ceftriaxone, cefotaxime, ceftazidime and aztreonam) or tazobactam (4 µg/ml) (piperacillin).

IEF. Cell extracts were obtained by ultrasonic treatment, and isoelectric focusing was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 9.5, as previously described (Bonnet *et al.*, 2001), with IRT-2 (pI 5.2), TEM-1 (5.4), TEM-2 (pI 5.6), OXA-1 (pI 7.4), SHV-1 (pI 7.6), CTX-M-15 (pI 8.9) and AmpC (pI 9.2) as standards.

Amplification, sequencing and cloning of β-lactamase genes. The β-lactamase-encoding genes were detected and sequenced, as described elsewhere (Mendonça *et al.*, 2006a). The complete SHV-encoding open reading frame of the bla_{SHV-1} and bla_{SHV-72} genes was amplified with the specific primers SHVsf and SHVsr (Mendonça *et al.*, 2006a). The bla_{SHV-1} and bla_{SHV-72} genes were cloned as follows: proofreading Isis DNA polymerase (Qbiogene, Irvine, CA) was used for bla_{SHV-72} and proofreading iProofTM high-fidelity DNA polymerase (Bio-Rad Laboratories Inc., Hercules, CA) was used for bla_{SHV-1}. PCR products were ligated in the *Sma*I site of the plasmid pBK-CMV, and the recombinant plasmids were electroporated in *E. coli* DH5α Δ*ampC*. The transformants harboring the recombinant SHV-encoding plasmids (pBK-SHV-72 and pBK-SHV-1) were selected on Mueller-Hinton agar supplemented with 30 µg/ml kanamycin and 16 µg/ml ticarcillin. The sequence and the orientation of the inserted open reading frames were determined from PCR experiments, which were performed with different combinations of primers pBK-CMV1' (5'-CTAGTGGATCCAAAGAATTCAAAAAGC-3'), pBK-CMV2' (5'-AATTGGGTACACTTACCTGGTACCC-3'), SHVsf and SHVsr.

β-Lactamase preparation. The SHV-producing clones were grown for 18 h at 37ºC in 6 liters of Luria-Bertani broth complemented with yeast extract, 30 µg/ml of kanamycin and 16 µg/ml of ticarcillin. After centrifugation, bacterial pellets were suspended with MES-NaOH 20mM (pH 6) and disrupted by ultrasonic treatment as previously described (Bonnet *et al.*, 2000a). The extract was then clarified by centrifugation and treated with DNase I (Roche Applied Science, Meylan, France). Purification was carried out by ion-exchange chromatography with an SP Sepharose column or HiPrep 16/10 SP HF column (Amersham Pharmacia Biotech) and gel filtration chromatography with a Superose 12 or HiPrep 16/60 Sephacryl S-100 HR column (Amersham Pharmacia Biotech), using a fast-protein liquid chromatography system as previously described (Bonnet *et al.*, 2001). The protein concentration was estimated by the BCA protein assay kit (Pierce, Rockford, IL,). The purity

of enzymes was estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (Bonnet *et al.*, 2001).

Determination of β-lactamase kinetic constants. The K_m and k_{cat} of the SHV-1 and SHV-72 enzymes were obtained with purified extracts by a computerized microacidimetric method, using 702 SM Titrino pHstat apparatus (Labia *et al.*, 1973). These kinetic parameters were determined by the analysis of the complete hydrolysis time-courses and the kinetic progress curves were fitted by nonlinear least-squares regression (Labia *et al.*, 1973). The IC_{50}s (clavulanate and tazobactam) were determined as described elsewhere (Bonnet *et al.*, 2001), with 200 µM ticarcillin as reporter substrate. The kinetic constants were determined three times for each subtract tested.

MDS. The model of the mutant enzyme (SHV-72) was constructed on the basis of SHV-1 crystal structure (Kuzin *et al.*, 1999). SHV-72 and SHV-1 enzymes were solvated with water in a periodic cubic box that was large enough to contain the system and 1 nm of solvent on all sides. Version 1.8.2 of the VMD package was used to manipulate the two systems (Humphrey *et al.*, 1996). The GROMACS software package, version 3.2 (Lim *et al.*, 2001), and the geometric and charge parameters of the OPLSAA (optimized potentials for liquid simulations in all-atom) force field (Jorgensen *et al.*, 1996) were used to carry out all energy minimizations and MDSs. TIP3P parameters were used for the water molecules (Jorgensen *et al.*, 1983). The particle-mesh Ewald method was used to treat long-range electrostatics (Darden *et al.*, 1993). All covalent bond lengths were constrained by the SHAKE algorithm (Ryckaert *et al.*, 1977) with a relative tolerance of 10^{-4}. The systems were equilibrated as reported previously (Maveyraud *et al.*, 2002) and MDSs of 400 ps were then made with a time step of 1.5 fs and coordinates collected every 0.0015 ps. The velocities of all atoms were generated from a Maxwellian distribution. The temperature was kept constant at 300 K, while the pressure was kept constant by the weak coupling constant of 1 bar using Berendsen's algorithms (Lindahl *et al.*, 2001).

Nucleotide sequence accession number. The new *bla*$_{SHV}$ nucleotide sequence was submitted to the EMBL Nucleotide Sequence Database as *bla*$_{SHV-72}$ with accession number AM176547.

RESULTS

Phenotypic characterization. The clinical *K. pneumoniae* INSRA1229 strain exhibited resistance to amoxicillin (2,048 µg/ml), ticarcillin (512 µg/ml) and piperacillin (32 µg/ml) (Table 4.2). The strain was susceptible to cephalosporins, monobactams, imipenem, ciprofloxacin, gentamicin and trimethoprim. *K. pneumoniae* INSRA1229 was also resistant to amoxicillin combined with clavulanic acid (64 µg/ml). MIC for piperacillin-tazobactam was much lower than that for amoxicillin-clavulanic acid combination (Table 4.2).

Table 4.2 - MICs of β-lactam antibiotics for the clinical *K. pneumoniae* strain INSRA1229, SHV-72 and SHV-1-producing transformants and the recipient *E. coli* DH5α Δ*ampC*

Antimicrobial drug	MIC (µg/mL) for strains:			
	E. coli DH5α Δ*ampC*	*E. coli* DH5α-SHV-1[a]	*E. coli* DH5α-URA1229[b]	*K. pneumoniae* INSRA1229
Amoxicillin	8	2,048	512	2,048
Amoxicillin + CLA[c]	8	8	64	64
Ticarcillin	4	1,024	8	512
Piperacillin	2	64	4	32
Piperacillin + TAZ[d]	1	2	2	2
Mecillinam	0.125	0.5	0.25	0.25
Cephalothin	8	16	4	8
Cefuroxime	4	4	8	2
Cefoperazone	≤0.25	4	≤0.25	2
Ceftriaxone	0.03	0.03	0.03	0.03
Ceftriaxone+ CLA	0.06	0.03	0.03	≤0.015
Cefotaxime	0.06	0.06	0.06	0.125
Cefotaxime + CLA	0.06	0.06	0.06	0.06
Ceftazidime	0.25	0.5	0.25	0.06
Ceftazidime + CLA	0.125	0.25	0.25	0.06
Aztreonam	0.06	0.125	0.125	≤0.015
Aztreonam + CLA	0.06	0.125	0.125	≤0.015
Cefepime	0.03	0.03	0.03	0.03
Cefoxitin	4	8	8	4
Imipenem	0.25	0.25	0.125	0.125
Ciprofloxacin	≤0.125	≤0.125	≤0.125	≤0.125
Gentamicin	≤0.125	0.25	0.25	0.5
Trimethoprim	≤0.125	≤0.125	≤0.125	0.5

[a] *E. coli* DH5α-SHV-1 was the transformant producing SHV-1.
[b] SHV-72-producing *E. coli* DH5α-URA1229 was the transformant corresponding to *K. pneumoniae* INSRA1229.
[c] CLA, clavulanic acid at a fixed concentration of 2 µg/ml.
[d] TAZ, tazobactam at a fixed concentration of 4 µg/ml.

Molecular characterization of *bla*$_{SHV-72}$ gene. The *bla*$_{SHV}$ gene of INSRA1229 was amplified and sequenced. The sequence showed the nonsynonymous nucleotide mutations A10T, C425T and A689G compared to *bla*$_{SHV-1}$. According to Ambler numbering (Ambler, 1980), these mutations lead to the two previously described amino acid substitutions Ile8Phe and Ala146Val, and to the new substitution Lys234Arg. The corresponding enzyme was designated SHV-72 and its gene was cloned downstream of promoter *LacZ* of the plasmid pBK-CMV, as well as *bla*$_{SHV-1}$. The SHV-72-producing clone, designated *E. coli* DH5α-URA1229, exhibited a β-lactam resistance phenotype similar to that of the clinical strain (Table 4.2).

Biochemical proprieties of β-lactamase SHV-72. The clinical strain and the corresponding clone produced only β-lactamases of pI 7.6, which is compatible with the amino acid sequence of SHV-72. SHV-72 and SHV-1 were purified from the *E. coli* clones by ion exchange and gel filtration. The rate of purity was estimated to be ≥ 96% for SHV-72 and ≥ 95% for SHV-1 on SDS-PAGE gel as a band of 28kDa, which corresponded to the molecular mass deduced from the amino acid sequence (data not shown).

The kinetic parameters for SHV-72 were determined for seven β-lactams and compared with those of SHV-1 (Table 4.3). k_{cat} values of SHV-72 against penicillins were higher than those of SHV-1, and K_m values were comparable or slightly higher (19 to 43 µM *versus* 11 to 31 µM). Overall, catalytic efficiency against penicillins was slightly higher for SHV-72 (k_{cat} /K_m, 36 to 286 $\mu M^{-1}.s^{-1}$) than for SHV-1 (k_{cat} /K_m, 20 to 84 $\mu M^{-1}.s^{-1}$), except for piperacillin (k_{cat} /K_m, 45 $\mu M^{-1}.s^{-1}$ versus 62 $\mu M^{-1}.s^{-1}$). In contrast with penicillins, SHV-72 had lower catalytic efficiency against cephalothin (11-fold) than SHV-1. Neither SHV-1 nor SHV-72 exhibited catalytic activity against oxyimino β-lactams.

Table 4.3 - Kinetic parameters of SHV-72 and SHV-1

Antibiotic	SHV-1			SHV-72		
	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Penicillin G	23 ± 0.42	1,937 ± 82	84.2 ± 2.0	19 ± 0.92	5,438 ± 176	286.2 ± 23.5
Amoxicillin	31 ± 1.29	1,044 ± 10	33.7 ± 1.1	26 ± 6.97	5,876 ± 79	226.0 ± 66.4
Ticarcillin	11 ± 3.40	220 ± 49	20.0 ± 1.7	36 ± 3.45	1,279 ± 24	35.5 ± 2.8
Piperacillin	24 ± 0.53	1,490 ± 96	62.1 ± 2.7	43 ± 5.45	1,918 ± 33	44.6 ± 6.5
Cephalothin	40 ± 1.46	128 ± 33	3.2 ± 0.8	141 ± 12.5	38 ± 7	0.3 ± 0.1
Ceftazidime	ND[a]	<0.1	ND	ND	<0.1	ND
Cefotaxime	ND	<0.1	ND	ND	<0.1	ND

[a] ND, Not determinable.

The IC_{50} for SHV-1 and SHV-72 are as follows: for clavulanic acid, the IC_{50} was 0.17 µM for SHV-1 and 1.72 µM for SHV-72; for tazobactam, the IC_{50} was 0.11 µM for SHV-1 and 0.08 µM for SHV-72. The IC_{50} of clavulanic acid was 10-fold higher for SHV-72 than for SHV-1, and IC_{50} of tazobactam was similar for both enzymes. Tazobactam was 22-fold more active than clavulanic acid against SHV-72 enzyme.

MDS. The enzyme SHV-72 was modeled from the crystallographic structure of SHV-1 (Kuzin *et al.*, 1999). The behaviors of SHV-72 and SHV-1 were compared during MDSs of 400 ps at a temperature of 300 K. The MDS for each model were checked for stability by monitoring several overall properties such as the radius of gyration, the secondary structure, root mean squared deviation (RMSD) from the initial structure, and the kinetic and potential energies (data not shown). These parameters were found to stabilize after about 100 ps, and hence, data from 300 ps were used for all subsequent analyses. The radius of gyration and the RMSDs of Cα atoms were similar to those for the crystallographic structure of SHV-1 (Table 4.4). The secondary structure was also preserved during the simulation (Table 4.4). The relatively small deviations were further evidence of the inherent stability of the model and indicated that the dynamic structures of the models remained in the realm of the crystal SHV-1 geometry during the course of the simulation. The largest fluctuations were localized in loops connecting the secondary structure elements, as is usual in MDSs of proteins (data not shown). The introduction of Ala146Val and Lys234Arg substitutions caused no overall or large-scale deviation of the dynamic properties.

Table 4.4 - Summary of statistical data for 300ps MDSs

Enzyme and use	Radius of gyration (Å)[a]	Cα RMSD (Å)	Secondary structure analysis (%)			
			Helix	β-sheet	Coil	Turn
Molecular modeling:						
- SHV-72	1.81 ± 0.01	0.88 ± 0.12[b]	41.5 ± 2.7	16.7 ± 0.8	18.2 ± 0.8	14.6 ± 1.8
- SHV-1	1.80 ± 0.00	0.90 ± 0.16[b]	42.8 ± 2.6	17.2 ± 0.4	18.2 ± 0.6	12.7 ± 1.4
Crystal structure:						
- SHV-1[c]	1.79	1.20 ± 0.11[d]	44.5	17.0	17.3	13.2

[a] Average radius of gyration of nonhydrogen atoms.
[b] RMSD of α-carbon atoms with respect to the minimized starting structures.
[c] Crystal structure of β-lactamase SHV-1 (PDB identification code 1SHV) (Kuzin *et al.*, 1999).
[d] RMSD of α-carbon atoms with respect to the molecular dynamics structures.

Position 146, which is located at the surface of the protein and at distance from the catalytic site (distance between Cα of positions 70 and 140 : 16.7 Å), did not modify the positioning of surrounding residues. Residue Arg234 is located in the catalytic site and adopted the conformation observed in the crystallographic structure of the class A β-lactamase PSE-4 (Figure 4.1A) (Lim *et al.*, 2001). Overall, the architecture of the active site

was identical for both enzymes SHV-72 and SHV-1. All residues of the active site except residue Ser130 had similar positioning in SHV-1 and SHV-72.

The behavior of Ser130 side chain was different in the two enzymes. In the initial set of MDSs, χ1 angle of Ser130 was around -145° in the starting models (Figure 4.1A, Figure 4.2A and C), as observed in the crystal structure of class A enzymes such as the TEM-, SHV- and CTX-M-type enzymes (Chen *et al.*, 2005; Kuzin *et al.*, 1999; Minasov *et al.*, 2002). After 130 ps of MDS, Ser130 χ1 angle of SHV-72 increased to -61 ± 11° and thereafter remained stable until the end of the simulation (Figure 4.2A). In SHV-1, χ1 angle kept the value of -145 ± 12° during almost all the simulation (Figure 4.2C).

A B

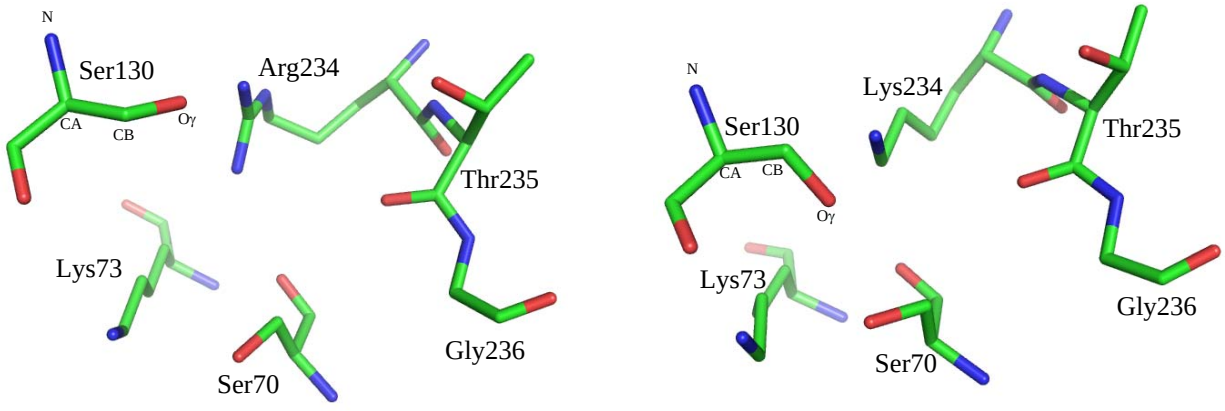

Figure 4.1 - The two conformations of Ser130 side chain. (A) SHV-72 exhibiting Ser130 χ1 angle value of -145°. (B) SHV-1 exhibiting Ser130 χ1 angle value of -60°. χ1 is the angle between the plane containing the atoms N, CA, CB and the plane containing the atoms CA, CB and Oγ.

In a second set of MDS, χ1 angle of Ser130 was set at -61° in the starting models by manual modeling (Figure 4.1B, Figure 4.2B and D). For SHV-72, χ1 angle value of Ser130 was -67 ± 10° and stable (Figure 4.2B). In contrast, the Ser130 χ1 angle of SHV-1 decreased after 160 ps from -58 ± 10° to -153 ± 20° and remained stable until the end of the MDS (Figure 4.2D). The Lys234Arg substitution thus stabilized an atypical conformation of the Ser130 side chain. This conformation was characterized by an χ1 angle between -50° and -77°, which moved the Oγ atom of Ser130 around 3.5 Å away from the key Oγ atom of the reactive serine (Ser70).

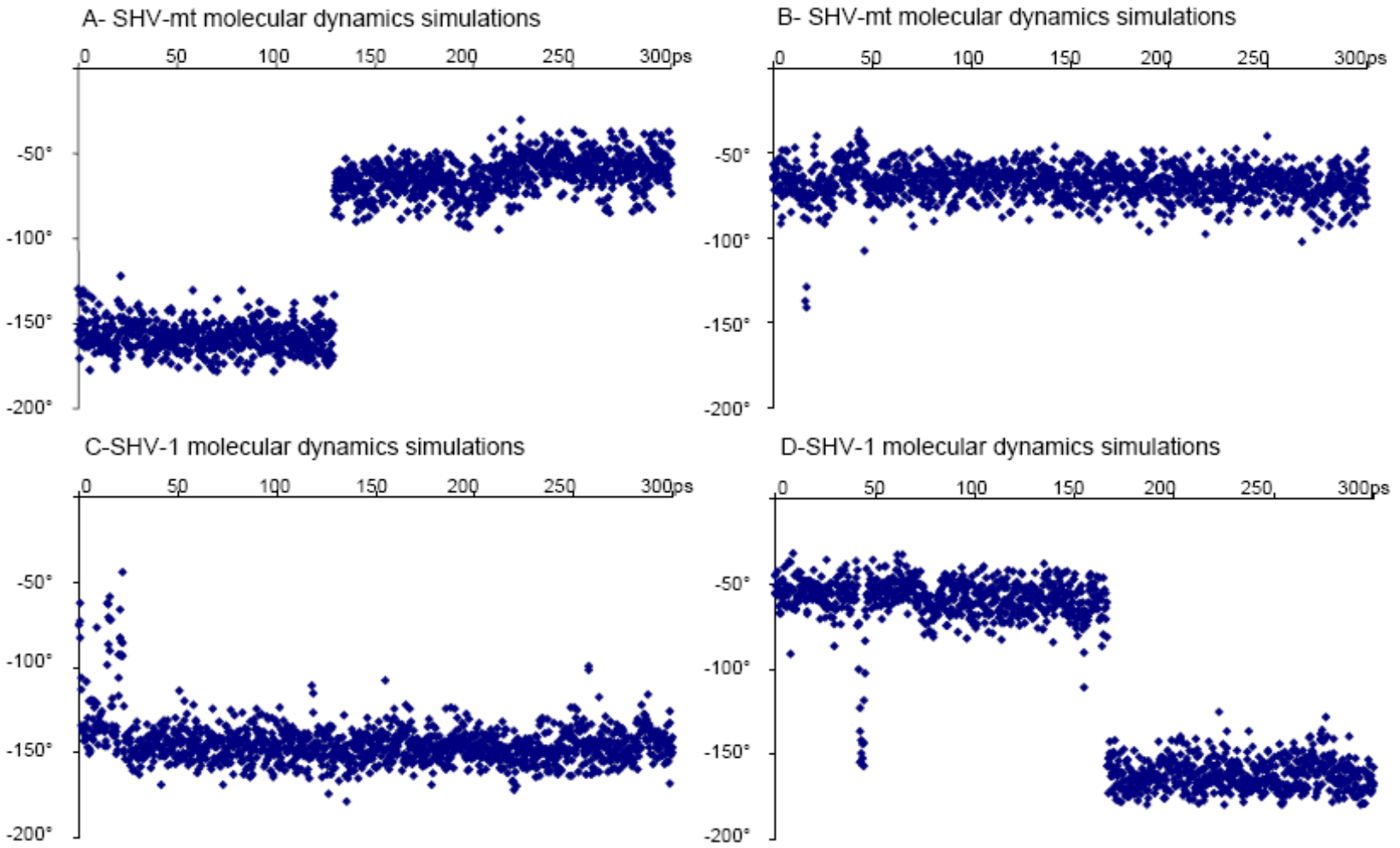

Figure 4.2 - χ1 angle of residue Ser130 during 300ps molecular dynamics simulations. (A) SHV-72 with a starting χ1 angle of -148°; (B) SHV-72 with a starting χ1 angle of -60°; (C) SHV-1 with a starting χ1 angle of -148°; (D) SHV-1 with a starting χ1 angle of -60°

DISCUSSION

The study of β-lactamases that are resistant to inhibitors is of great importance owing to the restricted number of drugs capable of eluding bacterial resistance. In this work we characterized a new enzyme, SHV-72, from a *K. pneumoniae* isolated in a Portuguese hospital, that exhibits unusual resistance to amoxicillin and the amoxicillin-clavulanic acid combination. SHV-72 harbored three substitutions: Ile8Phe in the leader peptide, Ala146Val at distance from the catalytic pocket (distance between Cα atoms 146 and 70: ≈17 Å) and the substitution Lys234Arg in the catalytic pocket. To our knowledge, this last substitution is observed for the first time in a natural SHV/TEM-type β-lactamase (Lenfant *et al.*, 1991).

The resulting new enzyme induced resistance phenotype compatible with that of an inhibitor-resistant penicillinase. No effect was observed on imipenem MIC, beside the presence of the substitution Ala146Val (Poirel *et al.*, 2003). The kinetic constant confirmed a high catalytic efficiency against penicillins, no significant activity against oxyimino β-lactams, and decreased susceptibility to clavulanate in comparison with results for SHV-1.

Only the three SHV-type enzymes SHV-10, SHV-26 and SVH-49 have been previously described as resistant to inhibitors. In contrast to SHV-72, these enzymes have an increase in K_m values and/or a decrease in k_{cat} values against penicillins (Chang *et al.*, 2001; Dubois *et al.*, 2004; Prinarakis *et al.*, 1997). In SHV-10 and SHV-49, the amino acid substitutions Ser130Gly and Met69Ile reduced activity against β-lactam substrates (Dubois *et al.*, 2004; Prinarakis *et al.*, 1997). The introduction of Lys234Arg in TEM-1 by site-directed mutagenesis experiments induce a 10-fold decrease of the affinity against penicillins (Lenfant *et al.*, 1991). The mutations observed in SHV-72 did not significantly affect K_m values and did not decrease catalytic activity against penicillins.

Among IRSs, SHV-10 is the most resistant (with an IC_{50} 41-fold higher than that for SHV-1) to inhibitors and SHV-26 the least (with an IC_{50} 3-fold higher than that for SHV-1) (Chang *et al.*, 2001; Prinarakis *et al.*, 1997). SHV-72 like SHV-49 (Dubois *et al.*, 2004), was 10-fold more resistant to clavulanic acid than SHV-1. The Ser130Gly substitution in SHV-10 also induces resistance to inhibitors in TEM-, OXY- and CTX-M-type enzymes (Aumeran *et al.*, 2003; Leflon-Guibout *et al.*, 2000b; Prinarakis *et al.*, 1997; Sirot *et al.*, 1998). The mechanism of inhibition by clavulanic acid is based on the formation of a covalent cross-link between Oγ atoms of Ser70 and Ser130 by residual atoms of the inhibitor. In enzymes harboring Gly130, this residue, which is deprived of the side chain, prevents cross-linking with position 70 (Sulton *et al.*, 2005; Sun *et al.*, 2004; Thomas *et al.*, 2005). In SHV-49, the substitution Met69Ile is responsible for resistance to inhibitors (Dubois *et al.*, 2004).

By analogy with inhibitor-resistant TEMs (Wang *et al.*, 2002), substitution in position 69 may decrease the susceptibility to inhibitors because of the modification of Ser130 side-chain positioning. In SHV-72, the Lys234Arg substitution is probably responsible for resistance to inhibitors owing to its location in the catalytic pocket and in the vicinity of residue 130.

To understand the role of Arg234 in the resistance to clavulanic acid, SHV-72 was modeled from SHV-1 crystal structure (Kuzin *et al.*, 1999) and analyzed during MDSs. SHV-72 and SHV-1 models exhibited different behavior only in the local region of residue 234. In Lys234-harboring class A β-lactamases, the conformation of the Ser130 side chain is such that its $\chi 1$ values are in the range of -120.5°to -163.5°, as for SHV-1 (-140°). In SHV-72 MDSs, an alternative conformation of the Ser130 side chain ($\chi 1 \approx$ -64°) appeared because of a hydrogen bond with Arg234, as previously observed in the crystal structure of the Arg234-harboring enzyme PSE-4 (Lim *et al.*, 2001). This alternative conformation is probably stabilized because of the restricted ability of Arg234 to move and establish hydrogen bond, since hydrogen bonds are favorable only in the plane of the rigid arginine guanidium group. This conformation is probably involved in the weak susceptibility to inhibitors of SHV-72.

The change of $\chi 1$ angle by around -64° moved the Ser130 Oγ atom away from the reactive Ser70 Oγ atom. This movement of the Oγ of Ser130, which is the ultimate covalent attachment point for the inhibitors, may therefore prevent the cross-link. Such a resistance mechanism has been previously proposed for TEM-32, for which the substitution Met69Ile induces, by another mechanism, the same movement of Ser130 side chain ($\chi 1$ angle -64°) (Wang *et al.*, 2002). The effects of substitutions Met69Ile, Ser130Gly and Lys234Arg therefore share a common logic for inhibitor resistance.

The Oγ atom of Ser130 plays a role in the hydrolysis of β-lactams (Lamotte-Brasseur *et al.*, 1994). In SHV-72, this role could presumably be disrupted. However, the enzyme did not lose its catalytic efficiency. This behavior may be explained by the coexistence of the two Ser130 conformers and/or the replacement of Ser130 Oγ atom by a water molecule, as observed in the crystal structures of the enzymes deprived of Ser130 such as SHV-10 and TEM-76 (Sun *et al.*, 2004; Thomas *et al.*, 2005).

In conclusion, we report a new SHV-type penicillinase resistant to clavulanic acid. The resistance to clavulanic acid is induced by substitution Lys234Arg, which probably affects the positioning of Ser130 side chain, a key element of the inhibition reaction mediated by clavulanic acid.

CHAPTER V

Molecular epidemiology and antimicrobial susceptibility of extended- and broad-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolated in Portugal

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ABSTRACT

All 187 *K. pneumoniae* strains isolated over six consecutive months of 1999 in 17 Portuguese health institutions were studied: 89% were resistant to ampicillin, 31% to trimethoprim-sulphametoxazole, 17% to aminoglycosides and 3% to fluoroquinolones; 16% were multidrugresistant and 14% expressed an ESBL phenotype confirmed by genotyping. Molecular methods identified 11 strains possessing $bla_{\text{ESBL-SHV}}$ genes ($bla_{\text{SHV-2A}}$, $bla_{\text{SHV-5}}$, $bla_{\text{SHV-12}}$ and $bla_{\text{SHV-55}}$), nine with $bla_{\text{ESBL-TEM}}$ ($bla_{\text{TEM-3}}$, $bla_{\text{TEM-10}}$ and $bla_{\text{TEM-24}}$) and seven with $bla_{\text{GES-1}}$, encoding ESBL enzymes; 160 with $bla_{\text{SHV-1}}$ and $bla_{\text{SHV-type}}$ encoding non-ESBL enzymes. Overall, we detected 15 new β-lactamases: SHV-60 to SHV-62, SHV-71 and SHV-73 to SHV-83. The genetic relatedness of 108 isolates was studied by pulsed-field gel electrophoresis (PFGE) analysis. The strains were diverse, and 18 clusters were defined, the largest including 12 strains of different biological origins, and six of which expressed GES-1 enzymes. Twenty additional strains isolated during a second period, between March and November 2006, in three of the participating hospitals, contained ESBL-encoding genes whereas none of the strains isolated in the same hospitals in 1999 carried such genes: $bla_{\text{SHV-5}}$, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-10}}$, $bla_{\text{TEM-52}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-32}}$ and $bla_{\text{CTX-M-61}}$ (firstly described in the country). In this period we detected three new enzymes: SHV-106 to SHV-108. We provide evidence that the genotypes of *K. pneumoniae* strains is changing toward the emergence of ESBL enzymes.

INTRODUCTION

SHV β-lactamases are classified in groups 2b and 2be of Bush-Jacoby-Medeiros classification scheme and in Ambler class A (Ambler *et al.*, 1991; Bush *et al.*, 1995). These enzymes are constitutive of the majority of *K. pneumoniae* strains. The chromosomal $bla_{\text{SHV-1}}$ gene encodes resistance to amoxicillin and was believed to be ubiquitous in *K. pneumoniae* (Babini & Livermore, 2000); these genes can be associated with plasmid-borne β-lactamases (Paterson & Bonomo, 2005), and if plasmid-mediated the possibility of development of an ESBL phenotype increases (Hammond *et al.*, 2008). Co-expression of SHV enzymes and ESBL, from TEM, CTX, or other SHV enzyme families is common (Romero *et al.*, 2005). ESBLs have a high clinical and epidemiological importance since they lead to therapeutic failure and increase hospital costs (Lee *et al.*, 2006a; Peterson, 2008).

Little is known about the antimicrobial resistance mechanisms of *K. pneumoniae* in Portugal. *K. pneumoniae* strains are found worldwide associated to pneumonia and urinary tract infections in nosocomial and community environments (Bell *et al.*, 2007; Caccamo *et al.*, 2006; Zhanel *et al.*, 2005). We report phenotypic and genotypic analysis of clinical isolates of

K. pneumoniae of both community and nosocomial origin recovered in various hospitals and public health institutions in the country.

MATERIALS AND METHODS

Setting and bacterial isolates. A total of 187 unduplicated *K. pneumoniae* strains collected consecutively in 17 Portuguese public health institutions (from which 14 were hospitals) in three different regions of Portugal (North, Centre and Lisbon and Tagus Valley), over a six-month period from the first of January of 1999, were sent to the Antibiotic Resistance Unit at the NIH in Lisbon. The bacteriology laboratories of these public health institutions are participating in a surveillance program with NIH, as contributors to the Antibiotic Resistance Surveillance Program in Portugal (ARSIP): one *K. pneumoniae* isolate and the corresponding laboratory records from each patient were sent to NIH. Origin of infection (nosocomial or community acquired) was identified according to Centers of Disease Control and Prevention criteria (Garner *et al.*, 1996).

Twenty *K. pneumoniae* strains, also collected consecutively, but in a second period, between March and November of 2006, in three of the hospitals (North, Centre, and Lisbon and Tagus Valley regions), were included as a prospective laboratory surveillance for comparison regarding ESBL production.

Each isolate was identified with the API 20E System. Strains used as control for both PCR and IEF were the same as previously (Mendonça *et al.*, 2007).

Antibiotic susceptibility testing. MICs of 23 antibiotics were determined by microdilution broth method (MicroScan Panel Sólo 1S, Dade Behring, West Sacramento, California, USA) against strains of both periods (187 from 1999 and 20 from 2006). Results were interpreted according to CLSI guidelines (2007). An isolate was considered multidrug-resistant if it had reduced susceptibility to three or more structurally unrelated antibiotics. For strains producing new enzymes and their respective transconjugants, MICs were also determined by an agar dilution method, according to the SFM (Cavallo *et al.*, 2007); MICs of amoxicillin, ceftriaxone, cefotaxime, ceftazidime and aztreonam were determined alone and combined with a given concentration of clavulanic acid (2 µg/ml). The 187 strains were tested by the Etest ESBL method using strips with cefotaxime and ceftazidime both alone and associated with clavulanate.

IEF. Cell extracts from the 187 isolates were obtained by ultrasound treatment and IEF was used to determine the pIs of all β-lactamases as previously (Caniça *et al.*, 1997a). The pI of each β-lactamase was compared with those of enzymes produced by control strains.

PCR, gene sequencing and transfer of resistance. PCR amplification and sequencing was performed for all strains with specific primers to search for and to identify bla_{TEM}, bla_{OXA}, bla_{SHV}, bla_{CTX-M} and a*mpC* β-lactamase genes, as previously described (Mendonça *et al.*, 2007). The primer SHVi (5'-GCT GGT TTA TCG CCG ATA AGA C-3') was used allowing the complete sequence of the bla_{SHV} gene to be determined. Specific primers were design for PCR amplification and sequencing of the bla_{GES} gene (GESf, 5'-TTC CAT CTC AAG GGA TCA CC-3' and GESr, 5'-GCG TCA ACT ATT TGT CCG TG-3'). Transference of ESBL encoding genes and of new bla_{SHV} resistance genes was tested, as previously (Mendonça *et al.*, 2006a).

PFGE. DNA preparation, PFGE and gel analysis for 108 strains from the first period of the study were performed as previously (Mendonça *et al.*, 2007). We selected strains for PFGE analysis according to the genotype: nine strains expressing ESBL enzymes of the SHV family, eight with ESBL enzymes of the TEM family, seven expressing ESBL enzymes of the GES family and 84 expressing SHV non-ESBL. The Dice band-based similarity coefficient, with a band position tolerance of 1.0% and an optimization of 1.0%, was used for clustering. A cutoff value of 80% similarity was determined by the cluster cutoff method according to Bionumerics software (Version 3.5; Applied Maths, Sint-Martens-Latem, Belgium). Isolates with a Dice band-based similarity coefficient value of >80% were considered to belong to the same cluster.

Statistical analysis. Analysis of variance was used to assess the significance of differences between various factors (sex, age group, specimen, region, multidrugresistance and local of acquired infection) and antibiotics, mostly from different classes (ampicillin, cephalothin, ceftazidime, gentamicin, ciprofloxacin and trimethoprim-sulphamethoxazole). Data were compared using χ^2 or Fisher's exact tests (SPSS software, version 14). For all tests, $P < 0.05$ was considered significant.

Nucleotide sequence accession numbers. The new bla_{SHV} sequences were submitted to the EMBL Nucleotide Sequence Database under the accession numbers AJ866283 (bla_{SHV-60}), AJ866284 (bla_{SHV-61}), AJ866285 (bla_{SHV-62}), AM176546 (bla_{SHV-71}), AM176548 (bla_{SHV-73}), AM176549 (bla_{SHV-74}), AM176550 (bla_{SHV-75}), AM176551 (bla_{SHV-76}), AM176552 (bla_{SHV-77}), AM176553 (bla_{SHV-78}), AM176554 (bla_{SHV-79}), AM176555 (bla_{SHV-80}), AM176556 (bla_{SHV-81}), AM176557 (bla_{SHV-82}), AM176558 (bla_{SHV-83}), AM941847 ($bla_{SHV-106}$), AM941848 ($bla_{SHV-107}$) and AM941849 ($bla_{SHV-108}$).

RESULTS

Clinical isolates and patient characteristics. Fifty-six of the 187 strains (from first period of the study) (30%) were identified as nosocomial, 57 (30%) were community acquired, and 74 (40%) were of unknown origin. One-hundred-and-twenty-six strains were isolated from urine (67%), 12 from wounds (6%), 18 from blood (10%), seven from sputum (4%), two from bronchoalveolar lavage (1%) and five from secretions (3%). Thirteen were from other biological samples, and four were of unknown origin. Of the 101 strains isolated from women, 33 were from patients over 60 years old, while of the 81 strains from men, 27 were from patients over 60 years old.

Identification of *bla* genes. PCR with bla_{SHV} primers identified 187 (100%) isolates carrying bla_{SHV} genes among the total strains collected in the first semester of 1999. All of these genes were sequenced: 11 possessed $bla_{ESBL\text{-}SHV}$ genes frequently associated with non-ESBL genes (including five $bla_{SHV\text{-}2A}$ of which four were associated with bla_{TEM} genes, one $bla_{SHV\text{-}5}$ plus $bla_{SHV\text{-}12}$ and five $bla_{SHV\text{-}55}$ of which one was associated with bla_{TEM}), nine $bla_{ESBL\text{-}TEM}$ ($bla_{TEM\text{-}3}$, $bla_{TEM\text{-}10}$, $bla_{TEM\text{-}24}$ all associated with non-ESBL *bla* genes), seven $bla_{GES\text{-}1}$ (associated with non-ESBL *bla* genes) and 160 non-ESBL bla_{SHV} genes associated or not with bla_{TEM} or bla_{OXA} genes (Table 5.1).

Strains isolated in a hospital (North region) in 2006 possessed genes encoding ESBL enzymes including $bla_{SHV\text{-}12}$, $bla_{TEM\text{-}52}$ and $bla_{CTX\text{-}M\text{-}32}$; isolates from another hospital (Centre region) had $bla_{SHV\text{-}5}$, $bla_{TEM\text{-}10}$ and $bla_{CTX\text{-}M\text{-}15}$; and isolates from a third hospital (Lisbon and the Tagus Valley) carried $bla_{SHV\text{-}12}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}61}$ genes (Table 5.2).

Antibiotic susceptibility. Twenty-seven strains were ESBL producers (14%). All ESBL-producing strains were resistant to ampicillin, ticarcillin, piperacillin and cefazolin; 30% had intermediate resistance to amoxicillin-clavulanate and 48% to piperacillin-tazobactam combinations; 7% were resistant to cefotaxime and 78% to ceftazidime. For other antibiotic families, 74% of the ESBL-producing strains were resistant to gentamicin and 89% to trimethoprim-sulphametoxazole (Table 5.3). For the β-lactam family, 88% of non-ESBL producers were resistant to ampicillin and 0.6% to amoxicillin-clavulanate (Table 5.3). All non-ESBL producers were susceptible to third generation cephalosporins.

Table 5.1 - Characterization of 187 strains (one per patient) isolated from January to June 1999, in 17 public health institutions in Portugal

Hospital code[a]	Region[b]	Non-ESBL enzymes				ESBL enzymes				Total
		Enzyme (no. of strains)	Specimen[c]	PFGE	Antibiotype[d]	Enzyme (no. of strains/no. of transconjugants)	Specimen[c]	PFGE	Antibiotype[d]	
A	Nr	SHV-1 (2)	B/U		(GEN) (TOB) (SXT)	SHV-5 + SHV-12 (1/1)	BL			12
		SHV-11 (3)	U/W	0056						
		SHV-28 (1)	U	0055	CIP NOR OFX SXT	SHV[e] + GES-1 (1/1)	U	0053	GEN TOB SXT	
		SHV-80 (1)	U							
		SHV (3)[e]	U	0053	(SXT)					
B	Nr	SHV-11 (1)	S		SXT	SHV-55 + TEM-1 (1/0)	F	0051	GEN TOB SXT	10
		SHV-28 (2)	U		(CIP) (NOR) (OFX) (SXT)					
		SHV-62 (5)	F/O/U			SHV-55 (1/0)	U	0051	SXT	
C	Nr	SHV-60 (2)	U		(GEN) (TOB) (SXT)					4
		SHV-61 (1)	U		GEN TOB SXT					
		SHV-62 (1)	U							
D	Nr	SHV-1 (1)	S	0039		SHV-55 (3/0)	O	0051, 0052	(GEN) (TOB) (SXT)	7
		SHV-11 (1)	U		SXT					
		SHV-81 (1)	U		SXT					
		SHV-82 (1)	U							
E	Nr	SHV-28 (2)	U	0012, 0057	SXT					4
		SHV-72 (1)	U	0014	GEN TOB					
		SHV-76 (1)	U	0048						
F	Cn	SHV-1 (12)	B/O/U	0028, 0032, 0049		SHV-28 + TEM-10 (4/3)	U	0054	GEN TOB SXT	37
		SHV-11 (9)	B/U/na	0016, 0023, 0037, 0040	(CIP) (NOR) (OFX) (GEN) (TOB) (SXT)					
		SHV-27 (1)	B							
		SHV-28 + (TEM)[e] (4)	B/U	0054	(GEN) (TOB) SXT					
		SHV-38 (2)	U	0038		SHV-1 + TEM-24 (1/1)	U	0058	TOB SXT	
		SHV-78 (1)	U							
		SHV-83 (1)	W			SHV-11 + TEM-24 (1/1)	U	0029	GEN SXT	
		SHV (1)[e]	U							

Continued

Table 5.1 - Continued

Hospital code[a]	Region[b]	Non-ESBL enzymes				ESBL enzymes				Total
		Enzyme (no. of strains)	Specimen[c]	PFGE	Antibiotype[d]	Enzyme (no. of strains/no. of transconjugants)	Specimen[c]	PFGE	Antibiotype[d]	
G	Cn	SHV-28 (1)								1
H	LTv	SHV-1 (1)	U	0005	SXT					2
		SHV-26 (1)	U							
I	LTv	SHV-1 (3)	B/U/W	0045, 0047		SHV-2A + TEM-1 (1/1)	BL	0042	GEN TOB SXT	18
		SHV-11 (6)	BL/U/W	0001, 0011, 0020						
		SHV-28 (2)	U							
		SHV-36 (1)	W			SHV-1 + TEM-3 (1/0)	U		TOB SXT	
		SHV-60 (1)	W	0033						
		SHV-74 (2)	U/W	0042, 0043						
		SHV-83 (1)	U	0034						
J	LTv	SHV-1 (1)	U	0003						2
		SHV-27 (1)	U	0017	SXT					
K	LTv	SHV-1 (3)	B/U							5
		SHV-83 (1)	U							
		SHV (1)[e]	U							
L	LTv	SHV-1 (4)	O/U	0006,0035, 0046						12
		SHV-11 (1)	U	0025						
		SHV-28 (2)	U		(CIP) (NOR) (OFX) SXT					
		SHV-71 (1)	U	0036						
		SHV-75 (1)	U	0024						
		SHV-77 (1)	U	0060						
		SHV (2)[e]	U	0008	SXT					

Continued

Table 5.1 - Continued

Hospital code[a]	Region[b]	Non-ESBL enzymes				ESBL enzymes				Total
		Enzyme (no. of strains)	Specimen[c]	PFGE	Antibiotype[d]	Enzyme (no. of strains/no. of transconjugants)	Specimen[c]	PFGE	Antibiotype[d]	
M	LTv	SHV-1 (3)	B/S/U	0019, 0053, 0060	(SXT)	SHV-2A + (TEM)[e] (4/3)	S/U/W	0029, 0042, 0043	(GEN) SXT	43
		SHV-11 (10)	U/P/B	0021, 0022, 0023, 0027						
		SHV-27 (2)	BL/O	0009						
		SHV-33 (1)	U			SHV-1 + TEM-10 (2/2)	U	0006, 0010	(AMK) GEN TOB SXT	
		SHV-62 (1)	B	0004						
		SHV-72 (3)	B/O/S	0014	(GEN) (TOB)					
		SHV-73 (1)	O	0014	GEN TOB					
		SHV-74 (3)	B/S/U	0042	(SXT)	SHV [e] + GES-1 (5/3)	O/U/W	0051, 0053	GEN TOB SXT	
		SHV-79 (1)	W							
		SHV (7)[e]	B/U	0044, 0053, 0060, 0061,	(GEN) (SXT)					
N	LTv	SHV (6)[e]	S/U	0002, 0013, 0062, 0063						6
O	LTv	SHV-1 + TEM-1A (3)	BL/U/W	0031, 0035, 0064	(GEN) TOB					8
		SHV-11 (2)	F/U	0010, 0018						
		SHV-28 (1)	U	0065						
		SHV-36 (1)	U	0030						
		SHV (1)[e]	U	0050						
P	LTv	SHV-1 (4)	U	0063		SHV [e] + GES-1 (1/0)	U	0053	GEN TOB SXT	13
		SHV-11 (3)	O/U	0026						
		SHV-28 (2)	U	0054, 0059	(SXT)					
		SHV-60 (1)	U	0041						
		SHV (2)[e]	U	0007, 0053	(SXT)					
Q	LTv	SHV-77 (1)	B	0015						3
		SHV-83 (1)	B							
		SHV (1)[e]	U							

[a] All hospitals were general hospitals, excepting B and O that are paediatric; D, H and J are other public health institutions.
[b] Cn, Centre region; LTv, Lisbon and Tagus Valley region; Nr, North region.
[c] B, blood; BL, bronchoalveolar lavage; F, feces; O, others; S, sputum; U, urine; W, wounds.
[d] Antibiotype for other families of antibiotics than β-lactams.
[e] More than one sequence present.
na, not available

Twenty-one % of the strains were resistant to trimethoprim-sulphametoxazole, 7% to gentamicin and 3% to the fluoroquinolones tested (Table 5.3). The only class of β-lactams to which all strains were susceptible was to carbapenems. Overall, 30 strains (16%) were multidrug resistant.

The percentage of isolates susceptible to different antibiotics (ampicillin, cephalothin, ceftazidime, gentamicin, trimethoprim-sulphametoxazole and ciprofloxacin) did not differ according to sex, age, specimen from which strains were isolated, region, or type of infection (community- or hospital-acquired) (data not shown).

Table 5.2 - Phenotypes and genotypes identified in 20 strains isolated in three hospitals (C, G, L), during a second period of the study (for comparison of ESBL production)

Hospital code (region)[a,b]	β-Lactamase	Antimicrobial resistance pattern[c]	No. of strains	Total
C	SHV-11 + TEM-52	AMP AZT CTX CPD CAZ CXM CFZ PIP TIC CIP GEN TOB SXT	1	10
(Nr)	SHV-106 + CTX-M-32	AMP AZT CTX CPD CAZ CXM CFZ CPE PIP TIC GEN TOB SXT	1	
	SHV-106 + TEM-1	AMP CTX CPD CXM CFZ PIP TIC GEN TOB SXT	1	
	SHV-1 + CTX-M-32	AMP AZT CTX CPD CXM CFZ CPE PIP TIC	1	
	SHV-12	AMP AZT (CTX) CPD (CAZ) (CXM) CFZ (CPE) PIP TIC CIP (GEN) (TOB) SXT	5	
	SHV-106	AMP CPD CFZ PIP TIC GEN TOB SXT	1	
G	SHV-108 + TEM-10 + CTX-M-15	AMP AZT CPD CAZ CFZ CPE PIP TIC GEN TOB	1	5
(Cn)	SHV-5	AMP AZT CPD CAZ CFZ PIP TIC GEN TOB SXT	1	
	SHV-5 + TEM-1 + CTX-M-15	AMP AZT CPD CAZ CFZ PIP TIC TOB SXT	1	
	SHV-1 + TEM-10 + CTX-M-15	AMP AZT CPD CAZ PIP TIC GEN TOB	1	
	SHV-107	AMP CPD CAZ CXM CFZ PIP TIC	1	
L	SHV-1 + TEM-1 + CTX-M-15 + OXA[d]	AMP AZT CTX CPD CXM CFZ CPE PIP TIC GEN TOB SXT	1	5
(LTv)	SHV-1 + CTX-M-15 + OXA[d]	AMP AZT CTX CPD CAZ CXM CFZ CPE PIP TIC GEN TOB SXT	1	
	SHV-11 + CTX-M-61	AMP AZT CTX CPD CXM CFZ CPE PIP TIC CIP GEN TOB SXT	2	
	SHV-12 + TEM-1	AMP AZT CPD CAZ CXM CFZ PIP TIC CIP GEN TOB SXT	1	

[a] All hospitals were general hospitals (same hospital codes of Table 5.1).
[b] Cn, Centre region; LTv, Lisbon and Tagus Valley region; Nr, North region.
[c] AMP, ampicillin; AZT, aztreonam; CTX, cefotaxime; CPD, cefpodoxime; CAZ, ceftazidime; CXM, cefuroxime; CFZ, cefazolin; CPE, cefepime; PIP, piperacillin; TIC, ticarcillin; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; SXT, trimethoprim-sulfamethoxazole.

Table 5.3 - MIC_{50}, MIC_{90}, range and percentage of non-susceptible (%IR) and resistant (%R) of ESBL and non-ESBL producers, among 187 *K. pneumoniae* isolates recovered in Portugal

Antimicrobial Agent	ESBL producers (n=27)					Non-ESBL producers (n=160)					CLSI Guidelines[b] (µg/ml)	
	MIC_{50}	MIC_{90}	Range	Susceptibility[a] % IR	Susceptibility[a] % R	MIC_{50}	MIC_{90}	Range	Susceptibility[a] % IR	Susceptibility[a] % R	S	R
Ampicillin	>16	>16	>16	100.0	100.0	>16	>16	≤4 - >16	94.4	87.5	≤8	≥32
Ampicillin-Sulbactam	>16/8	>16/8	≤8/4 - >16/8	81.5	77.8	≤8/4	>16/8	≤8/4 - >16/8	40.0	31.9	≤8/4	≥32/16
Amoxicillin-Clavulanic acid	8/4	16/8	≤4/2 - 16/8	29.6	0.0	≤4/2	8/4	≤4/2 - >16/8	6.9	0.6	≤8/4	≥32/16
Piperacillin	>64	>64	>64	100.0	100.0	32	>64	≤16 - >64	52.5	41.3	≤16	≥128
Piperacillin-Tazobactam	≤16	>64	≤16 - >64	48.1	25.9	≤16	≤16	≤16 - 64	8.1	3.1	≤16/4	≥128/4
Ticarcillin	>64	>64	>64	100.0	100.0	>64	>64	≤8 - >64	98.8	91.9	≤16	≥128
Cephalothin	>16	>16	≤8 - >16	92.6	92.6	≤8	>16	≤8 - >16	23.8	12.5	≤8	≥32
Cefazolin	>16	>16	>16	100.0	100.0	≤1	16	≤1 - >16	10.6	5.6	≤8	≥32
Cefuroxime	>16	>16	8 - >16	81.5	55.6	≤2	8	≤2 - >16	5.6	1.9	≤8	≥32
Cefotaxime	8	32	1 - > 32	44.4	7.4	≤0.5	≤0.5	≤0.5 - 1	0.0	0.0	≤8	≥64
Ceftriaxone	16	>32	≤2 - > 32	70.4	14.8	≤2	≤2	≤2	0.0	0.0	≤8	≥64
Aztreonam	>16	>16	≤1 - > 16	51.9	51.9	≤1	≤1	≤1 - 8	0.0	0.0	≤8	≥32
Ceftazidime	>16	>16	1 - > 16	77.8	77.8	≤0.5	≤0.5	≤0.5 - 8	0.0	0.0	≤8	≥32
Ceftazidime-Clavulanic acid	≤0.5	≤0.5	≤0.5 - 2	0.0	0.0	≤0.5	≤0.5	≤0.5	0.0	0.0	-	-
Cefepime	1	>16	≤0.5 - > 16	18.5	14.8	≤0.5	≤0.5	≤0.5 - 8	0.0	0.0	≤8	≥32
Cefoxitin	≤4	≤4	≤4	0.0	0.0	≤4	≤4	≤4 - > 16	5.6	2.5	≤8	≥32
Imipenem	≤0.5	≤0.5	≤0.5 - 1	0.0	0.0	≤0.5	≤0.5	≤0.5 - 1	0.0	0.0	≤4	≥16
Meropenem	≤1	≤1	≤1	0.0	0.0	≤1	≤1	≤1	0.0	0.0	≤4	≥16
Amikacin	≤8	32	≤8 - > 32	11.1	3.7	≤8	≤8	≤8 - 16	0.0	0.0	≤16	≥64
Gentamicin	>8	>8	≤2 - >8	74.1	74.1	≤2	≤2	≤2 - >8	7.5	6.9	≤4	≥16
Tobramycin	>8	>8	≤2 - >8	66.7	66.7	≤2	≤2	≤2 - >8	6.9	6.9	≤4	≥16
Ciprofloxacin	≤0.5	≤0.5	≤0.5	0.0	0.0	≤0.5	≤0.5	≤0.5 - >2	3.8	3.1	≤1	≥4
Norfloxacin	≤2	≤2	≤2 - 4	0.0	0.0	≤2	≤2	≤2 - >8	3.8	3.1	≤4	≥16
Ofloxacin	≤0.5	2	≤0.5 - 2	0.0	0.0	≤0.5	2	≤0.5 - >4	3.1	3.1	≤2	≥8
Trimethoprim-sulphamethoxazole	>2/38	>2/38	≤1/19 - >2/38	88.9	88.9	≤1/19	>2/38	≤1/19 - >2/38	21.3	21.3	≤2/38	≥4/76

[a] IR, non-susceptible; R, resistant.
[b] MIC values (µg/ml) interpreted as: S, susceptible; R, resistant; The values between S and R are intermediate.

Strains producing new SHV β-lactamases. Fifteen strains expressing new β-lactamases identified during the first period of the study presented resistance to amoxicillin, and some presented resistance to the combination amoxicillin plus clavulanic acid (INSRA1234, INSRA1677 and INSRA2500) (Table 5.4). Only two of these strains (INSRA3530 and INSRA1234) expressed another β-lactamase (TEM), which was detected either by PCR or IEF. None of the new β-lactamases were classified as ESBL either by agar dilution or Etest analysis. Three strains of the second period of the study expressed new enzymes: SHV-106 and SHV-107, both identified as probable ESBL, and SHV-108, which was not possible to clarify the corresponding phenotype (because it was associated to TEM-10 and CTX-M-15 enzymes). The non-synonymous nucleotide mutations and aminoacid substitutions in the new bla_{SHV} genes are reported in Table 5.5.

Transfer of antibiotic resistance. Overall, it was possible to transfer ESBL encoding genes in 59% of all ESBL *K. pneumoniae*-producing strains (Table 5.1). Only one of the 18 clinical isolates producing new SHV enzymes gave a transconjugant. This transconjugant (*E. coli* C600-INSRA577) carried the bla_{SHV-60} gene, which presented antibiotic susceptibility phenotype similar to that of the clinical strain (*K. pneumoniae* INSRA577) (Table 5.4).

Clonality of K. pneumoniae strains. PFGE analysis was used to establish the genetic relatedness of 108 of the *K. pneumoniae* strains producing four different types of β-lactamases (see material and methods). Sixty-five PFGE profile types were identified; 47 included only a single isolate (non-clustered) genetically unrelated to other isolates in the study. The remaining 18 profile types, defined clusters I to XVIII, included two or more isolates that were genetically related (with >80% similarity) or indistinguishable (with 100% identity) (Table 5.1). The largest cluster XIV (include profile type 0053) consisted of 12 strains producing mainly SHV β-lactamases, co-expressed with GES-1 in six cases; these strains were of different biological origins, isolated in two hospitals in Lisbon and the Tagus Valley region and one in the North region. The second largest cluster XV (include profile type 0054) consisted of only strains collected from urine (n=7); all expressed SHV-28 and most (n=4) co-expressed TEM-10 enzymes; six were isolated in one hospital in the Centre region. (Table 5.1). Strains with multidrugresistance were detected in eight clusters (II, III, VIII, IX, XII, XIII, XIV XV) and in two unique profiles types with a single clone (profile type 0055, 0058).

Table 5.4 - Agar dilution MIC of antibiotics for clinical *K. pneumoniae* strains producing new SHV enzymes, one transconjugant and recipient

Strain no.	MIC (µg/mL)													pI	No. of new Enzyme	Accession no.
	AML	AMC	CF	CPZ	CRO	CRO/CA	CTX	CTX/CA	CAZ	CAZ/CA	AZT	AZT/CA	FOX			
E. coli C600 Nal	4	4	8	≤0.25	0.03	0.03	0.06	0.03	0.125	0.125	0.06	0.03	8			
INSRA577	128	4	2	0.5	≤0.016	≤0.016	≤0.016	≤0.016	0.06	0.06	0.25	0.03	4	7.6	SHV-60	AJ866283
E. coli C600-INSRA577[a]	≥128	8	8	1	0.03	0.03	0.06	0.03	0.125	0.125	0.06	0.06	8	7.6	SHV-60	
INSRA3530[b]	>4,096	16	8	2	0.125	0.06	0.125	≤0.016	0.25	0.25	0.06	0.06	4	5.4 ; 7.6	SHV-61	AJ866284
INSRA698	>4,096	16	8	32	0.125	0.03	≤0.016	≤0.016	0.25	0.06	0.06	0.03	2	7.4	SHV-62	AJ866285
INSRA229	64	4	2	≤0.25	0.03	≤0.016	0.06	≤0.016	0.06	0.06	0.03	≤0.016	2	7.6	SHV-71	AM176546
INSRA1234[b]	>4,096	≥128	8	4	0.06	0.03	0.03	0.03	0.125	0.06	0.03	0.03	2	5.4 ; 7.6	SHV-73	AM176548
INSRA2267	>4,096	16	16	1	0.125	0.06	0.125	0.06	0.5	0.25	0.125	0.06	16	7.6	SHV-74	AM176549
INSRA1488	128	≤ 2	4	≤0.25	0.06	0.06	0.06	0.03	0.125	0.125	0.06	0.03	4	7.6	SHV-75	AM176550
INSRA1677	512	≥128	4	0.5	0.06	0.06	0.06	0.03	0.25	0.25	0.06	0.06	4	7.6	SHV-76	AM176551
INSRA2500	>4,096	32	8	0.5	0.5	0.06	0.125	0.03	0.25	0.25	0.125	0.06	8	7.6	SHV-77	AM176552
INSRA2607	128	≤2	4	0.5	0.06	0.06	0.125	0.06	0.25	0.125	0.03	0.03	4	7.6	SHV-78	AM176553
INSRA3018	128	≤2	4	0.5	0.06	0.06	0.125	0.06	0.125	0.125	0.03	0.03	4	7.6	SHV-79	AM176554
INSRA3644	4,096	16	16	4	0.125	0.06	0.06	0.03	0.5	0.125	0.06	0.06	8	7.6	SHV-80	AM176555
INSRA3768	32	≤2	4	≤0.25	0.06	0.03	0.125	0.06	0.125	0.125	0.03	≤0.016	4	7.6	SHV-81	AM176556
INSRA3774	64	≤2	2	≤0.25	0.03	0.03	0.06	0.06	0.125	0.06	≤0.016	≤0.016	2	7.6	SHV-82	AM176557
INSRA2270	>4,096	16	32	≤0.25	0.06	0.03	0.06	≤0.016	0.125	0.125	0.06	0.03	4	7.6	SHV-83	AM176558
INSRA6633[b]	≥128	≥128	1024	64	32	0.125	16	0.06	8	0.5	4	0.06	8	5.4 ; 7.6	SHV-106	AM941847
INSRA6884	≥128	≥128	256	64	128	0.5	4	0.25	128	8	4	0.25	4	7.6	SHV-107	AM941848
INSRA6896[c]	≥128	32	512	256	128	0.125	2	0.125	>256	1	>256	0.25	16	5.6 ; 7.6; 8.5	SHV-108	AM941849

AML, amoxicillin; AMC, amoxicillin plus clavulanic acid; CF, cephalothin; CPZ, cefoperazone; CRO, ceftriaxone; CRO/CA, ceftriaxone plus clavulanic acid; CTX, cefotaxime; CTX/CA, cefotaxime plus clavulanic acid; CAZ, ceftazidime; CAZ/CA, ceftazidime plus clavulanic acid; AZT, aztreonam; AZT/CA, aztreonam plus clavulanic acid; FOX, cefoxitin.

[a] *E. coli* C600-INSRA577 was a transformant of *K. pneumoniae* INSRA577 (both harbouring SHV-60 enzyme)

[b] Phenotype corresponding to the expression of the new enzyme plus TEM-1 enzyme.

[c] Phenotype corresponding to the expression of the new enzyme plus TEM-10 and CTX-M-15 enzymes.

Table 5.5 - Non-synonymous nucleotide mutations in 18 new *bla*$_{SHV}$ genes identified by sequencing in *K. pneumoniae* strains of Portuguese origin, and corresponding aminoacid substitutions

Gene	Nucleotide [a] (aminoacid)[b] at position no.																									Accession no.
	2 (5)	7 (7)	8 (7)	9 (7)	10 (8)	17 (10)	40 (18)	52 (22)	61 (25)	92 (35)	148 (54)	212 (75)	322 (112)	410 (141)	418 (144)	424 (146)	425 (146)	503 (172)	547 (187)	627 (213)	689 (234)	691 (235)	700 (238)	742 (253)	
bla$_{SHV-1}$	T (Met)	T (Tyr)	A (Tyr)	T (Tyr)	A (Ile)	T (Leu)	A (Thr)	G (Ala)	G (Ala)	T (Leu)	G (Gly)	T (Val)	C (His)	C (Thr)	G (Gly)	G (Ala)	C (Ala)	C (Ala)	G (Ala)	C (Asp)	A (Lys)	A (Thr)	G (Gly)	A (Asn)	AF148850
bla$_{SHV-60}$										A (Gln)									A (Thr)						AJ866283
bla$_{SHV-61}$						G (Arg)				A (Gln)															AJ866284
bla$_{SHV-62}$										A (Gln)			T (Tyr)												AJ866285
bla$_{SHV-71}$													T (Tyr)				T (Val)								AM178546
bla$_{SHV-73}$		Del	Del	Del													T (Val)				G (Arg)				AM176548
bla$_{SHV-74}$								A (Thr)		A (Gln)															AM176549
bla$_{SHV-75}$																								C (His)	AM176550
bla$_{SHV-76}$							G (Ala)			A (Gln)										A (Glu)					AM176551
bla$_{SHV-77}$										A (Gln)		C (Ala)													AM176552
bla$_{SHV-78}$											A (Ser)														AM176553
bla$_{SHV-79}$										A (Gln)								T (Val)							AM176554
bla$_{SHV-80}$										A (Gln)						A (Thr)									AM176555
bla$_{SHV-81}$										A (Gln)					A (Ser)										AM176556
bla$_{SHV-82}$					G (Val)				A (Thr)	A (Gln)															AM176557
bla$_{SHV-83}$	A (Lys)																								AM176558
bla$_{SHV-105}$			T (Phe)																				A (Ser)		AM941847
bla$_{SHV-107}$										A (Gln)												G (Ala)			AM941848
bla$_{SHV-108}$										A (Gln)				T (Ile)											AM941849

[a] Numbering is according to Sutcliffe (Sutcliffe, 1978).
[b] Numbering is according to Ambler (Ambler *et al.*, 1991).
Del, nucleotide deleted.

DISCUSSION

We conducted this study because there was little available information about antimicrobial susceptibility and resistance mechanisms of *K. pneumoniae* isolates in Portugal and this pathogen remain one of the major ESBL producing strains worldwide (Pitout & Laupland, 2008). In addition, an increase of hospital costs is associated to ESBL-producing *K. pneumoniae* (Lee *et al.*, 2006a). We studied the phenotype and genotype of 187 clinical *K. pneumoniae* strains responsible for community and hospital-acquired infections and isolated between January and June of 1999 in 14 Portuguese hospitals and three other public health institutions (representing three of the five regions of mainland Portugal).

K. pneumoniae isolates are expected to present an intrinsic resistance to ampicillin (Heritage *et al.*, 1999), but only 95% (178/187) of all our strains showed reduced susceptibility and 89% resistance to this antibiotic. Indeed, similar resistance have been reported for the same year elsewhere in Europe (84%) (Kahlmeter, 2003).

ESBL production was detected in 14% of the strains: this is a lower prevalence than reported for 1999 in paediatrics centres in France (32%) (Raymond *et al.*, 2007), in an Italian survey (20%) and in the Asia-Western Pacific region (18%) (Luzzaro *et al.*, 2006; Turnidge *et al.*, 2002); it is however higher than the 9% observed for North America (Sader *et al.*, 2005); 13% of our isolates from patients with urinary tract infections were ESBL-producing strains (data not shown), and the corresponding figures are 27%, 2% and 19% for Latin America, United States and Italy, respectively (Caccamo *et al.*, 2006; Turnidge *et al.*, 2002). None of our isolates from blood expressed ESBL, and this is contrary to findings in other countries (Tumbarello *et al.*, 2006). The differences in the prevalence of ESBL production between isolates in Portugal and elsewhere may be due to the more extensive use of other antibiotic families (penicillins, quinolones and macrolides) rather than third generation cephalosporins in Portugal (Goossens *et al.*, 2005).

Only 3% of the strains presented resistance to fluoroquinolones, but resistance to aminoglycosides and trimethoprim-sulphametoxazole was more widespread (19% and 30%, respectively). *K. pneumoniae* isolates resistant to these antibiotics are less common (1%, 0% and 12%, respectively) elsewhere in Europe (Kahlmeter, 2003). A multi-continental study reported similar resistance to aminoglycosides (13%) and to fluoroquinolones (8%) (Deshpande *et al.*, 2006).

Overall, of the 187 strains studied in six months of 1999, 27 possessed bla_{ESBL} genes. We identified nine strains which had $bla_{\text{ESBL-TEM}}$ genes including the $bla_{\text{TEM-10}}$ gene, the most prevalent within this group (five strains, 56%). In our survey, the $bla_{\text{TEM-10}}$ gene was present in two of 17 public health institutions in the Centre and Lisbon and Tagus Valley regions, one of which was previously associated to an endemic TEM-10 enzyme produced by *K. pneumoniae* strains, identified during a survey in 1995 and 1996 (Barroso *et al.*, 2000).

The *bla*$_{GES-1}$ gene, encoding an ESBL enzyme, was detected in three hospitals in two different regions (North and Lisbon and Tagus Valley); the *bla*$_{GES-1}$ gene has already been found in one hospital in the Lisbon and Tagus Valley region (Duarte *et al.*, 2003).

We document substantial genetic heterogeneity among *K. pneumoniae* strains collected in different regions in the same period of 1999. No epidemic strains could be defined, and this contrasts with the situation for *E. coli* for which a survey after 2004 including the same regions identified the presence of a CTX-M-15, OXA-30 and TEM-1 producing clone (Mendonça *et al.*, 2007). In the major profile type (0053) in our collection 12 of the 108 (11%) strains shared 80% similarity, and all 12 were isolated in three hospitals.

Of the strains isolated in three hospitals in the second period of the study (year 2006), 45% expressed CTX-M enzymes in contrast with only non-ESBL present in 1999 isolates; this confirms the emergence of this family of β-lactamases in *K. pneumoniae* in Portugal as in other countries (Cantón & Coque, 2006; Livermore *et al.*, 2007). Romero *et al* (2005) also did not detect any CTX-M enzymes in 1999, but 33% of strains isolated in 2002 carried these genes. In Italy, in 2003, only 12% of *K. pneumoniae* strains presented CTX-M enzymes (Mugnaioli *et al.*, 2006), and between 1998 and 2003, the SENTRY program in the Asia-Pacific region, reported that 25% of strains possessed CTX-M enzymes (Bell *et al.*, 2007). Emergence of this type has previously been observed in Portugal for *E. coli* and other *Enterobacteriaceae* strains (Machado *et al.*, 2007; Mendonça *et al.*, 2007).

This work documents the substantial diversity of β-lactamases produced by *K. pneumoniae* and identified several new β-lactamases. Among the 160 strains producing SHV non-ESBL enzymes in the first period, we identified 15 new *bla*$_{SHV}$ genes. The importance of ESBL as a serious clinical problem for treat patients with *K. pneumoniae* expressing these resistance determinants is of concern; however, the clinical importance of the non-ESBL enzymes is yet to be revealed (Paterson & Bonomo, 2005; Heritage *et al.*, 1999). A similar nationwide survey with a population based may elucidate the real emergence of other β-lactamases produced nowadays by *K. pneumoniae* strains in this country, as we show that CTX-M β-lactamases added to the list look to predominate.

CHAPTER VI

OKP and LEN β-lactamases produced by clinical *Klebsiella pneumoniae* strains in Portugal

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ABSTRACT

Of the 308 clinical *K. pneumoniae* strains collected in 21 Portuguese health institutions, eleven encoded for LEN and nine for OKP enzymes; of these, fifteen were new enzymes. Ninety-one % of LEN and all OKP producer strains were resistant to amoxicillin. We demonstrate that these β-lactamase producers were highly diverse.

TEXT

K. pneumoniae strains are responsible for pneumonia and urinary tract infections, both in community and hospital environments (Gonzalez-Vertiz *et al.*, 2001; Goossens *et al.*, 2005; Karlowsky *et al.*, 2001), and can be classified into three clusters according to the intrinsic beta-lactamases they express. More than 80% of strains belong to group KpI, which express SHV enzymes, while groups KpII and KpIII each account for 10% of the population and express OKP and LEN enzymes, respectively (Brisse & Verhoef, 2001; Hæggman *et al.*, 2004). Until now co-expression of LEN and OKP enzymes and ESBLs, from the TEM, CTX and SHV families, is rarely described (Romero *et al.*, 2005). The aim of this study was to identify and characterize strains expressing OKP and LEN enzymes among clinical *K. pneumoniae* isolates and to understand their distribution in Portugal.

A total of 308 *K. pneumoniae* strains isolated from patients, were collected in 21 health institutions in three different regions of Portugal (North, Centre and Lisbon and Tagus Valley), between August 1998 and October 1999 and between May 2004 and May 2005, and sent to the Antibiotic Resistance Unit at the NIH in Lisbon. The bacteriology laboratories of these hospitals are participating in a surveillance program with the NIH, as contributors to the ARSIP: they send one *K. pneumoniae* isolate from each patient and corresponding laboratory records.

The detection and identification of LEN and OKP enzymes produced by 20 of 308 *K. pneumoniae* strains were performed by PCR and sequencing. *E. coli* R111 (TEM-1, pI 5.4; bla_{TEM-1} plus *ampC*), *S. enterica* serovar Typhimurium (OXA-1, pI 7.4; bla_{OXA-1}), *E. coli* C600 (SHV-1, pI 7.6; bla_{SHV-1}) and *E. coli* UA1526 (CTX-M-15, pI 8.9; $bla_{CTX-M-15}$) were used as control strains for PCR. The bla_{LEN} and bla_{OKP} genes were identified and sequenced with bla_{SHV} primers, as previously described (Mendonça et al., 2007). The primer SHVi (5'-GCTGGTTTATCGCCGATAAGAC-3') was used allowing the complete sequence of the bla_{LEN} and bla_{OKP} genes to be determined. Transference of β-lactam resistance genes was tested for all strains presenting new bla_{OKP} and bla_{LEN} genes, as previously described (Mendonça *et al.*, 2006a).

MICs of non-β-lactam antibiotics (ciprofloxacin, norfloxacin, ofloxacin, amikacin, gentamycin, tobramycin, trimethoprim-sulphamethoxazole) were evaluated by a microdilution broth method (MicroScan Panel Sólo 1S) against LEN and OKP producer strains. Results of the susceptibility testing were interpreted according to CLSI guidelines (2007). MICs of β-lactam antibiotics (amoxicillin, piperacillin, cephalothin, cefuroxime, cefotaxime, ceftazidime and cefoxitin) were determined by an agar dilution method, according to the SFM (Cavallo *et al.*, 2007). MICs of amoxicillin, cefotaxime and ceftazidime were also determined in combination with 2 µg/mL of clavulanic acid. All strains were tested by the Etest ESBL method using strips with cefotaxime and ceftazidime both alone and associated with clavulanate.

Cell extracts from the 20 isolates were obtained by ultrasound treatment and IEF was used to determine the pIs of β-lactamases as previously described (Caniça *et al.*, 1997a). The pIs were compared with those of enzymes produced by the following control strains: *E. coli* R57b (OXA-3, pI 7.1), *E. coli* pMG48 (OXA-2, pI 7.7), *E. coli* INSRA5776 (CTX-M-14, pI 8.1); and those strains also used as control for PCR. PFGE was used to establish the genetic relatedness of all *K. pneumoniae* strains, as previously described (Mendonça *et al.*, 2007).

The majority of the 20 *K. pneumoniae* isolates were from women (60%), patients over 60 years-old (40%) and from urinary tract (70%). Overall, OKP producing strains (n=9) were resistant to amoxicillin, while only 11% were resistant to amoxicillin plus clavulanic acid combination and 11% were non-susceptible to both piperacillin and cephalothin (Table 6.1). Intermediate level of resistance for cefuroxime and cefoxitin was detected in 22% of the OKP-producing strains. LEN-producing strains presented lower resistance values to amoxicillin and piperacillin (91% and 9%, respectively), but showed 9% resistance to amoxicillin plus clavulanic acid combination (Table 6.1). No synergy with ceftazidime and the β-lactamase inhibitor was detected for either group, indicating that all strains were non-ESBL producers (Table 6.1); the Etest was negative confirming those results. All strains were susceptible to trimethoprim-sulphametoxazole, aminoglycosides and fluoroquinolones. The phenotypes of strains producing OKP and LEN enzymes were similar to the previously described for these types of β-lactamases (Fèvre *et al.*, 2005; Hæggman *et al.*, 2004).

IEF method identified that LEN β-lactamases had pIs between 7.3 and 7.6, and OKP β-lactamases between 7.0 and 7.8 (Table 6.1). PCR and sequencing identified 11 bla_{LEN} genes, seven of which were firstly describe here (LEN-18 to LEN-24) (Table 6.2); eight new bla_{OKP} genes were also identified: two coding to OKP-A enzymes (OKP-A-11 and OKP-A-12) and six coding to OKP-B enzymes (OKP-B-15 to OKP-B-20), according to the classification established by Fèvre *et al*. (Fèvre *et al.*, 2005) (Table 6.3).

Table 6.1 - Distribution, clinical features, and characteristics of clinical *K. pneumoniae* strains producing OKP and LEN enzymes

Strain no.	Region[a]	Origin of infection[b]	Sex (Patient Age)	Specimen	PFGE Profile	MIC (µg/mL)										β-lactamase	
						AML	AMC	PIP	CF	CXM	CTX	CTX/CA	CAZ	CAZ/CA	FOX	Type	pI
INSRA395	N	nd	Female (69 years)	Urine	0002	32	≤ 2	2	4	0.25	0.03	0.03	0.06	0.06	2	LEN-9	7.4
INSRA2943	N	Community Acq.	Male (33 years)	Other	0016	32	≤ 2	1	2	1	0.03	0.03	0.125	0.06	2	LEN-10	7.1
INSRA1864	LVT	Nosocomial	Female (18 days)	Urine	0006	64	4	8	4	2	0.03	0.03	0.125	0.125	4	LEN-11	7.4
INSRA578	LVT	Community Acq.	Male (76 years)	Urine	0011	64	4	8	4	4	0.06	0.06	0.125	0.125	2	LEN-18	7.4
INSRA1773	N	Community Acq.	80 (years)	Urine	0005	512	64	4	8	4	0.06	0.03	0.25	0.125	4	LEN-19	7.4
INSRA2941	N	Community Acq.	Male (8 months)	Urine	0004	64	≤ 2	8	2	4	0.06	0.06	0.25	0.25	1	LEN-20	7.4
INSRA2944	N	Nosocomial	Male (72 years)	Other	0004	64	≤ 2	8	2	4	0.06	0.03	0.25	0.125	2	LEN-20	7.4
INSRA3283	LVT	Nosocomial	Female (36 years)	Urine	0012	16	≤ 2	2	1	1	0.03	0.03	0.06	0.06	2	LEN-21	7.6
INSRA4364	LVT	nd	Female (nd)	Urine	0013	32	≤ 2	4	2	4	0.125	0.06	0.125	0.06	2	LEN-22	7.4
INSRA4473	LVT	Community Acq.	Female (73 years)	Blood	0008	32	≤ 2	4	2	4	0.03	0.03	0.125	0.125	4	LEN-23	7.4
INSRA5774	LVT	Nosocomial	Male (22 days)	Urine	0014	> 16	≤ 2	> 64	≤ 8	≤ 4	≤ 0.5	ND	≤ 0.5	≤ 0.5	≤ 4	LEN-24	7.4
INSRA13	LVT	Nosocomial	Female (78 years)	Blood	0009	1024	4	32	8	4	0.06	0.06	0.125	0.06	4	OKP-A-11	7.8
INSRA511	N	nd	Male (74 years)	Urine	0015	128	4	8	8	8	0.125	0.125	0.25	0.25	8	OKP-A-12	7.8
INSRA271	LVT	Community Acq.	Female (5 years)	Other	0001	256	128	16	1	1	≤ 0.016	≤ 0.016	0.125	0.06	1	OKP-B-15	7.4
INSRA377	N	nd	Male (64 years)	Urine	0003	128	≤ 2	2	0.06	0.25	≤ 0.016	≤ 0.016	0.125	0.125	1	OKP-B-16	7.4
INSRA2243	LVT	nd	Female (nd)	Urine	0010	64	≤ 2	4	2	2	0.06	0.03	0.06	0.06	2	OKP-B-17	7.4
INSRA4775	C	nd	Female (nd)	Urine	0017	64	≤ 2	4	2	4	0.06	0.03	0.125	0.06	4	OKP-B-17	7.4
INSRA2692	C	nd	Female (nd)	Pus	0017	128	4	16	32	16	0.5	0.25	0.5	0.25	32	OKP-B-18	7.0
INSRA4751	C	nd	Male (nd)	Urine	0018	64	≤ 2	8	4	4	0.06	0.06	0.125	0.125	4	OKP-B-19	7.3
INSRA4767	C	nd	Female (nd)	Urine	0007	256	4	16	8	16	0.25	0.25	0.5	0.25	16	OKP-B-20	7.3

AML, amoxicillin; AMC, amoxicillin plus clavulanic acid; PIP, piperacillin; CF, cephalothin; CXM, cefuroxime; CTX, cefotaxime; CTX/CA, cefotaxime plus clavulanic acid; CAZ, ceftazidime; CAZ/CA, ceftazidime plus clavulanic acid; FOX, cefoxitin.

[a] C, Centre; LVT, Lisbon and Tagus Valley; N, North.

[b] According to Centers of Disease Control and Prevention criteria (Garner *et al.*, 1996). Community Acq., community acquired

nd, data not available.

ND, Not determined.

The substitutions Asp88Val in LEN-21, Ile115Val in LEN-18, LEN-19 and LEN-20, Ala264Val in LEN-20 and Thr14Asn in LEN-24 were firstly described in this study. As in previous studies, we did not observe co-expression of SHV and LEN or OKP enzymes (Hæggman *et al.*, 2004; Remeli *et al.*, 2007; Siebor, *et al.*, 2005). Transfer experiments failed to produce transconjugants containing LEN or OKP enzymes, which may imply that the genes encoding for these β-lactamases are present in the chromosome or in non-transferable plasmids.

According to the PFGE analysis, all *K. pneumoniae* isolates were genetically diverse, presenting 18 different profile types. Only two clusters grouped isolates from the same hospital: cluster I included two strains from the North region producing LEN-20 enzymes and cluster II included two strains from the Center region, one producing an OKP-B-17 enzyme and another producing an OKP-B-18 enzyme (Table 6.1).

Table 6.2 - Amino acid substitutions in the seven new LEN enzymes identified by sequencing in *K. pneumoniae* strains of Portuguese origin

Gene	Nucleotide [a] (aminoacid)[b] at position no.											Accession no.
	41 (14)	58 (20)	250 (84)	263 (88)	328 (110)	329 (110)	343 (115)	589 (197)	667 (223)	769 (257)	791 (264)	
bla$_{LEN-2}$	C (Thr)	G (Asp)	S (Val)	A (Asp)	G (Val)	T (Val)	A (Ile)	G (Ala)	C (Pro)	C (Ile)	C (Ala)	AY037780
bla$_{LEN-18}$			G (Leu)		A (Thr)	C (Thr)	G (Val)			A (Leu)		AM850908
bla$_{LEN-19}$		T (Tyr)	G (Leu)		A (Thr)	C (Thr)	G (Val)					AM850909
bla$_{LEN-20}$			G (Leu)		A (Thr)	C (Thr)	G (Val)				T (Val)	AM850910
bla$_{LEN-21}$				T (Val)					G (Ala)			AM850911
bla$_{LEN-22}$								C (Pro)	G (Ala)			AM850912
bla$_{LEN-23}$		T (Tyr)	G (Leu)		A (Thr)	C (Thr)				A (Leu)		AM850913
bla$_{LEN-24}$	A (Asn)		G (Leu)									AM850914

[a] Numbering is according to Sutcliffe (Sutcliffe, 1978).
[b] Numbering is according to Ambler (Ambler *et al.*, 1991).

There are few studies about LEN and OKP enzymes. This work describes the phenotypic and genetic characterization of strains expressing OKP and LEN enzymes produced by clinical *K. pneumoniae* isolated in Portugal, among which 15 were new β-lactamases.

Nucleotide sequence accession number. The new *bla*$_{LEN}$ and *bla*$_{OKP}$ sequences were submitted to the EMBL Nucleotide Sequence Database under the accession numbers AM850908 (*bla*$_{LEN-18}$), AM850909 (*bla*$_{LEN-19}$), AM850910 (*bla*$_{LEN-20}$), AM850911 (*bla*$_{LEN-21}$), AM850912 (*bla*$_{LEN-22}$), AM850913 (*bla*$_{LEN-23}$), AM850914 (*bla*$_{LEN-24}$), AM850915 (*bla*$_{OKP-A-11}$), AM850916 (*bla*$_{OKP-A-12}$), AM850917 (*bla*$_{OKP-B-15}$), AM850918 (*bla*$_{OKP-B-16}$), AM850919 (*bla*$_{OKP-B-17}$), AM850920 (*bla*$_{OKP-B-18}$), AM850921 (*bla*$_{OKP-B-19}$), and AM850922 (*bla*$_{OKP-B-20}$).

Table 6.3 - Amino acid substitutions in the eight new OKP enzymes identified by sequencing in *K. pneumoniae* strains of Portuguese origin

Gene	Nucleotide [a] (aminoacid)[b] at position no.																									Accession no.
	56 (19)	71 (24)	85 (29)	89 (30)	91 (31)	92 (31)	100 (34)	143 (48)	155 (52)	176 (59)	275 (92)	386 (129)	410 (137)	550 (184)	575 (192)	578 (193)	580 (194)	632 (211)	687 (229)	742 (248)	787 (263)	793 (265)	804 (268)	823 (275)	
bla$_{OKP-A-1}$	T (Val)	C (Pro)	A (Ile)	C (Thr)	C (Arg)	G (Arg)	A (Ser)	T (Val)	C (Thr)	A (Asn)	G (Arg)	G (Ser)	G (Ser)	G (Ala)	G (Ser)	A (His)	A (Thr)	A (Gln)	A (Glu)	A (Asn)	C (Pro)	T (Ser)	G (Ala)	A (Arg)	AM051138
bla$_{OKP-A-11}$	C (Ala)		G (Val)					C (Ala)		G (Ser)							G (Ala)		T (Asp)			A (Thr)			AM850915
bla$_{OKP-A-12}$	C (Ala)	A (Gln)	G (Val)					C (Ala)		G (Ser)							G (Ala)		T (Asp)			A (Thr)			AM850916
bla$_{OKP-B-15}$		A (Gln)		A (Lys)	A (Ile)	T (Ile)		C (Ala)		G (Ser)	A (His)			A (Thr)	C (Thr)	C (Pro)	T (Ser)	G (Arg)	T (Asp)	G (Asp)	G (Ala)	A (Thr)	A (Val)	G (Gly)	AM850917
bla$_{OKP-B-16}$		A (Gln)		A (Lys)	A (Ile)	T (Ile)		C (Ala)		G (Ser)	A (His)	C (Thr)	T (Ile)	A (Thr)	C (Thr)	C (Pro)	T (Ser)	G (Arg)	T (Asp)	G (Asp)		A (Thr)		G (Gly)	AM850918
bla$_{OKP-B-17}$		A (Gln)		A (Lys)	A (Ile)	T (Ile)	G (Gly)	C (Ala)		G (Ser)	A (His)			A (Thr)	C (Thr)	C (Pro)	T (Ser)	G (Arg)	T (Asp)	G (Asp)		A (Thr)	A (Val)	G (Gly)	AM850919
bla$_{OKP-B-18}$		A (Gln)		A (Lys)	A (Ile)	T (Ile)	G (Gly)	C (Ala)		G (Ser)	A (His)		T (Ile)	A (Thr)	C (Thr)	C (Pro)	T (Ser)		T (Asp)	G (Asp)	G (Ala)	A (Thr)		G (Gly)	AM850920
bla$_{OKP-B-19}$		A (Gln)		A (Lys)	A (Ile)	T (Ile)	G (Gly)	C (Ala)	T (Met)	G (Ser)	A (His)	C (Thr)	T (Ile)	A (Thr)	C (Thr)	C (Pro)	T (Ser)	G (Arg)	T (Asp)	G (Asp)		A (Thr)	A (Val)	G (Gly)	AM850921
bla$_{OKP-B-20}$		A (Gln)		A (Lys)	A (Ile)	T (Ile)		C (Ala)		G (Ser)	A (His)	C (Thr)	T (Ile)	A (Thr)	C (Thr)	C (Pro)	T (Ser)	G (Arg)	T (Asp)	G (Asp)	G (Ala)	A (Thr)	A (Val)	G (Gly)	AM850922

[a] Numbering is according to Sutcliffe (Sutcliffe, 1978).
[b] Numbering is according to Ambler (Ambler *et al.*, 1991).

CHAPTER VII

Diversity of the *bla*$_{SHV}$ genes

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ABSTRACT

The diversity of *bla*$_{SHV}$ genes based on nucleotide synonymous mutations and the presence or absence of the nonsynonymous mutation T92A was investigated; these mutations may result in different nucleotide sequences, designated here as sequence frameworks. We considered *bla*$_{SHV}$ gene sequences determined at the National Institute of Health in Lisbon and others from various bacterial species in the Genbank database. The totality of the gene coding region was used to align all the sequences. Eighty-three different *bla*$_{SHV}$ gene sequence frameworks were detected based on the combination of synonymous nucleotide mutations at 39 different positions and the non-synonymous mutation T92A, which results in amino acid substitution Leu35Gln and allows to differentiate SHV-11 from SHV-1. Synonymous nucleotide mutations A402G, G705A and C786G, were the most frequent. Among the 297 *bla*$_{SHV}$ genes studied, 61 were identified as *bla*$_{SHV-11}$ presenting 20 different nucleotide sequence frameworks and 59 were *bla*$_{SHV-1}$ with 18 nucleotide sequence frameworks. The complex molecular diversity of *bla*$_{SHV}$ genes impelled us to propose a classification, based on the name of the *bla*$_{SHV}$ gene, the parental origin (*bla*$_{SHV-1}$ or *bla*$_{SHV-11}$) and the number of the variant (the last being v83).

INTRODUCTION

β-Lactamases are an important cause of resistance to β-lactam antibiotics. The SHV enzymes are distributed worldwide (Livermore, 1995; Bradford, 2001). SHV-1 is the most prevalent β-lactamase in *K. pneumoniae*, but it is also present as a plasmid-borne gene in various other species (Livermore, 1995). Non-synonymous nucleotide substitutions of the parental *bla*$_{SHV-1}$ gene led to the emergence of ESBL enzymes, which are responsible for higher levels of resistance to extended-spectrum cephalosporins (Paterson & Bonomo, 2005). Until now, 112 SHV enzymes have been identified but several of them do not code for ESBL enzymes (http://www.lahey.org/studies/). In addition, synonymous nucleotide substitutions and the presence or absence of the synonymous mutation T92A are found in *bla*$_{SHV}$ genes, resulting in different nucleotide sequences, which we designated here as sequence frameworks, even for genes coding for the same enzyme. Like the parental *bla*$_{TEM-1}$ and *bla*$_{TEM-2}$ genes, which differ by one point mutation responsible for the Gln39Lys amino acid substitution, the *bla*$_{SHV-1}$ and *bla*$_{SHV-11}$ genes differ by one non-synonymous mutation corresponding to the Leu35Gln substitution (Nüesch-Inderbinen *et al.*, 1997).

The aim of this study was to analyse the diversity of nucleotide sequences of 297 SHV-encoding genes. The analysis of this diversity allowed us to develop and propose a classification to differentiate the various *bla*$_{SHV}$ gene sequence frameworks.

MATERIAL AND METHODS

Computer analysis of DNA and amino acid sequences. The sequences of 215 *bla*$_{SHV}$ genes, previously determined in the Antibiotic Resistance Unit at the NIH, were investigated (unpublished results) (Mendonça *et al.*, 2006a); 82 other *bla*$_{SHV}$ gene sequences, not detected in that collection, were downloaded manually from the NCBI GenBank database (Table 7.1) or retrieved from scientific publications. BioNumerics software was used for sequence entry and manipulation for all sequences. The *bla*$_{SHV-1}$ gene sequence, as found in the Genbank database (accession number AF148850), was used as reference for the development of the framework classification. A nucleotide sequence of 861 bp from each *bla*$_{SHV}$ gene analysed in this study (beginning at nucleotide 1 of the coding region, according to numbering of Sutcliffe), was used for sequence alignments (Sutcliffe, 1978). Numbering system of Ambler was also used with regards to amino acid positions (Ambler *et al.*, 1991).

RESULTS AND DISCUSSION

Using the sequence of the *bla*$_{SHV-1}$ gene submitted in 1999 to GenBank (accession number AF148850) as reference, the characterization of the diversity of the 297 *bla*$_{SHV}$ genes studied, permitted the identification of 83 different *bla*$_{SHV}$ gene sequence frameworks (Table 7.2). These frameworks resulted from the combination of both synonymous nucleotide mutations at 39 different nucleotide positions and the T92A non-synonymous mutation. Among the synonymous variations, three appeared to be more frequent: A402G (90%), G705A (60%) and C786G (66%) (Table 7.2). Ten years ago, other authors reported that 59% of 34 *bla*$_{SHV}$ genes had synonymous mutation A402G and 9% G705A (Nüesch-Inderbinen *et al.*, 1997). Among the 83 *bla*$_{SHV}$ gene sequence frameworks that we identified, 45 were based on the parental gene *bla*$_{SHV-1}$, characterized by a T residue at position 92 resulting in amino acid Leu at position 35, while 38 were based on the *bla*$_{SHV-11}$ gene with the non-synonymous mutation T92A encoding amino acid substitution Leu35Gln.

The high diversity of the *bla*$_{SHV}$ gene family compelled us to define a classification based on the following principle: for gene *bla*$_{SHV-X-1v}$ or $_{-11v}$ (1 to 83), X is the gene number that has already been attributed to the *bla*$_{SHV}$ gene and corresponding enzyme (http://www.lahey.org/studies); 1 or 11 indicates that this gene derived from either *bla*$_{SHV-1}$ or *bla*$_{SHV-11}$ parental genes; the number attributed to “v” indicates the framework variant according to the order of appearance in GenBank and literature (from 1 to 83, until now).

Table 7.1 - *bla*$_{SHV}$ genes used in this study (n=101) as described in the NCBI database[a]

bla$_{SHV}$ genes	Accession No. (year)	ESBL[b]	Organism	*bla*$_{SHV}$ genes	Accession No. (year)	ESBL[b]	Organism
bla$_{SHV-1}$	AF148850 (1999)	-	*E. coli*	*bla*$_{SHV-46}$	AY210887 (2003)	+	*K. oxytoca*
bla$_{SHV-1}$	X98100 (1997)	-	*K. pneumoniae*	*bla*$_{SHV-48}$	AY263404 (2003)	-	*K. pneumoniae*
bla$_{SHV-1}$	X98099 (1997)	-	*K. pneumoniae*	*bla*$_{SHV-49}$	AY528718 (2004)	-	*K. pneumoniae*
bla$_{SHV-1}$	X98098 (1997)	-	*K. pneumoniae*	*bla*$_{SHV-50}$	AY288915 (2003)	-	*K. pneumoniae*
bla$_{SHV-1}$	AF462396 (2002)	-	*Acinetobacter* spp	*bla*$_{SHV-51}$	AY289548 (2003)	-	*K. pneumoniae*
bla$_{SHV-2}$	AF282921 (2001)	+	*Shigella flexneri*	*bla*$_{SHV-53}$	AY590467 (2004)	-	*K. pneumoniae*
bla$_{SHV-2a}$	AF462393 (2002)	+	*K. pneumoniae*	*bla*$_{SHV-55}$	AJ863560 (2004)	+	*K. pneumoniae*
bla$_{SHV-2a}$	L47119 (1990)	+	*Salmonella* spp	*bla*$_{SHV-56}$	AY352599 (2003)	-	*A.baumannii*
bla$_{SHV-2a}$	X98102 (1997)	+	*K. pneumoniae*	*bla*$_{SHV-57}$	AY223863 (2003)	-	*E. coli*
bla$_{SHV-5}$	AF550679 (2007)	+	*E. coli*	*bla*$_{SHV-59}$	AY790341 (2004)	-	*K. pneumoniae*
bla$_{SHV-5}$	AF462394 (2002)	+	*E. coli*	*bla*$_{SHV-60}$	AJ866283 (2004)	-	*K. pneumoniae*
bla$_{SHV-5}$	X98103 (1997)	+	*K. pneumoniae*	*bla*$_{SHV-61}$	AJ866284 (2004)	-	*K. pneumoniae*
bla$_{SHV-6}$	Y11069 (1997)	-	*K. pneumoniae*	*bla*$_{SHV-62}$	AJ866285 (2004)	-	*K. pneumoniae*
bla$_{SHV-7}$	U20270 (1995)	+	*E. coli*	*bla*$_{SHV-63}$	EU342351 (2007)	-	*K. pneumoniae*
bla$_{SHV-8}$	U92041 (1997)	+	*E. coli*	*bla*$_{SHV-64}$	DQ174304 (2005)	+	*K. pneumoniae*
bla$_{SHV-9}$	S82452 (1996)	+	*K. pneumoniae*	*bla*$_{SHV-65}$	DQ174305 (2005)	+	*K. pneumoniae*
bla$_{SHV-11}$	Y18299 (1999)	-	*Shigella dysenteriae*	*bla*$_{SHV-66}$	DQ174306 (2005)	+	*K. pneumoniae*
bla$_{SHV-11}$	X98101 (1997)	-	*K. pneumoniae*	*bla*$_{SHV-67}$	DQ174307 (2005)	+	*K. pneumoniae*
bla$_{SHV-11}$	AY293069 (2003)	-	*E. coli*	*bla*$_{SHV-69}$	DQ166779 (2005)	+	*K. pneumoniae*
bla$_{SHV-12}$	AY293070 (2003)	+	*E. coli*	*bla*$_{SHV-69}$	DQ174308 (2005)	+	*K. pneumoniae*
bla$_{SHV-13}$	AF164577 (2000)	+	*K. pneumoniae*	*bla*$_{SHV-70}$	DQ013287 (2005)	+	*E. cloacae*
bla$_{SHV-14}$	AF226622 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-71}$	AM176546 (2005)	-	*K. pneumoniae*
bla$_{SHV-15}$	AJ011428 (2001)	+	*E. coli*	*bla*$_{SHV-72}$	AM176547 (2005)	-	*K. pneumoniae*
bla$_{SHV-16}$	AF072684 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-73}$	AM176548 (2005)	-	*K. pneumoniae*
bla$_{SHV-18}$	AF132290 (2000)	+	*K. pneumoniae*	*bla*$_{SHV-74}$	AM176549 (2005)	-	*K. pneumoniae*
bla$_{SHV-19}$	AF117743 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-75}$	AM176550 (2005)	-	*K. pneumoniae*
bla$_{SHV-20}$	AF117744 (2001)	+	*K. pneumoniae*	*bla*$_{SHV-76}$	AM176551 (2005)	-	*K. pneumoniae*
bla$_{SHV-21}$	AF117745 (2001)	+	*K. pneumoniae*	*bla*$_{SHV-77}$	AM176552 (2005)	-	*K. pneumoniae*
bla$_{SHV-22}$	AF117746 (2001)	+	*K. pneumoniae*	*bla*$_{SHV-78}$	AM176553 (2005)	-	*K. pneumoniae*
bla$_{SHV-24}$	AB023477 (1999)	+	*E. coli*	*bla*$_{SHV-79}$	AM176554 (2005)	-	*K. pneumoniae*
bla$_{SHV-25}$	AF208796 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-80}$	AM176555 (2005)	-	*K. pneumoniae*
bla$_{SHV-26}$	AF227204 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-81}$	AM176556 (2005)	-	*K. pneumoniae*
bla$_{SHV-27}$	AF293345 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-82}$	AM176557 (2005)	-	*K. pneumoniae*
bla$_{SHV-28}$	AF299299 (2000)	-	*K. pneumoniae*	*bla*$_{SHV-83}$	AM176558 (2005)	-	*K. pneumoniae*
bla$_{SHV-28}$	AF538324 (2002)	-	*K. pneumoniae*	*bla*$_{SHV-85}$	DQ322460 (2005)	-	*K. pneumoniae*
bla$_{SHV-29}$	AF301532 (2000)	-	*K. pneumoniae*	*bla*$_{SHV-86}$	DQ328802 (2005)	+	*K. pneumoniae*
bla$_{SHV-30}$	AY661885 (2004)	+	*E. cloacae*	*bla*$_{SHV-89}$	DQ193536 (2005)	+	*K. pneumoniae*
bla$_{SHV-31}$	AY277255 (2003)	-	*K. pneumoniae*	*bla*$_{SHV-92}$	DQ836922 (2006)	-	*K. pneumoniae*
bla$_{SHV-32}$	AY037778 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-93}$	EF373969 (2007)	-	*K. pneumoniae*
bla$_{SHV-33}$	AY037779 (2001)	-	*K. pneumoniae*	*bla*$_{SHV-94}$	EF373970 (2007)	-	*K. pneumoniae*
bla$_{SHV-34}$	AY036620 (2001)	+	*E. coli*	*bla*$_{SHV-95}$	EF373972 (2007)	-	*Citrobacter freundii*
bla$_{SHV-35}$	AY070258 (2002)	-	*K. pneumoniae*	*bla*$_{SHV-96}$	EF373971 (2007)	-	*A. baumannii*
bla$_{SHV-36}$	AF467947 (2002)	-	*K. pneumoniae*	*bla*$_{SHV-97}$	EF373973 (2007)	+	*Enterococcus faecalis*
bla$_{SHV-37}$	AF467948 (2002)	-	*K. pneumoniae*	*bla*$_{SHV-101}$	EU155018 (2007)	-	*K. pneumoniae*
bla$_{SHV-38}$	AY079099 (2003)	-	*K. pneumoniae*	*bla*$_{SHV-102}$	EU024485 (2007)	+	*E. coli*
bla$_{SHV-40}$	AF535128 (2002)	-	*K. pneumoniae*	*bla*$_{SHV-103}$	EU032604 (2007)	-	*K. pneumoniae*
bla$_{SHV-41}$	AF535129 (2002)	-	*K. pneumoniae*	*bla*$_{SHV-104}$	EU274581 (2007)	+	*K. pneumoniae*
bla$_{SHV-42}$	AF535130 (2002)	-	*K. pneumoniae*	*bla*$_{SHV-106}$	AM941847 (2008)	+	*K. pneumoniae*
bla$_{SHV-43}$	AY065991 (2003)	-	*K. pneumoniae*	*bla*$_{SHV-107}$	AM941848 (2008)	-	*K. pneumoniae*
bla$_{SHV-44}$	AY259119 (2003)	-	*K. pneumoniae*	*bla*$_{SHV-108}$	AM941849 (2008)	-	*K. pneumoniae*
bla$_{SHV-45}$	AF547625 (2002)	-	*K. pneumoniae*				

[a]Gene sequences unpublished and unsubmitted to GenBank database (and consequently, not included in this study): *bla*$_{SHV-4}$, *bla*$_{SHV-10}$, *bla*$_{SHV-23}$, *bla*$_{SHV-39}$, *bla*$_{SHV-47}$, *bla*$_{SHV-52}$, *bla*$_{SHV-54}$, *bla*$_{SHV-58}$, *bla*$_{SHV-68}$, *bla*$_{SHV-84}$, *bla*$_{SHV-87}$, *bla*$_{SHV-88}$, *bla*$_{SHV-90}$, *bla*$_{SHV-91}$, *bla*$_{SHV-98}$, *bla*$_{SHV-99}$, *bla*$_{SHV-100}$, *bla*$_{SHV-105}$; the *bla*$_{SHV-17}$ gene was not included because it was withdrawn from the site http://www.lahey.org/studies/webt.htm. Despite being unsubmitted the *bla*$_{SHV-3}$ gene sequence was included in the study as it has been published (Nicolas *et al.*, 1989).

[b]ESBL genotype based on the presence of non-synonymous mutations at nucleotide positions 700 and 703 and/or based on previous descriptions

Table 7.2 - Nucleotide sequence framework of 83 different *bla*$_{SHV}$ genes with the corresponding encoded β-lactamase

Framework	Nucleotide nb. (aminoacid nb.): 51 57 92 (35) 153 156 201 276 324 336 348 357 397 402 405 408 429 456 462 510 516 534 537 564 570 615 633 642 654 722 705 720 759 762 765 771 786 789 795 813 846	*bla*$_{SHV}$ gene	Encoded β-lactamase[b]
1v1[c]	G A T (Leu) C G C C C C C C C A G C A C C C C C G G G G G A C C G G C T G T C G C G G	*bla*$_{SHV-1-1v1}$; *bla*$_{SHV-2-1v1}$; *bla*$_{SHV-5-1v1}$; *bla*$_{SHV-6-1v1}$;*bla*$_{SHV-8-1v1}$; *bla*$_{SHV-19-1v1}$; *bla*$_{SHV-20-1v1}$; *bla*$_{SHV-21-1v1}$; *bla*$_{SHV-22-1v1}$; *bla*$_{SHV-49-1v1}$; *bla*$_{SHV-57-1v1}$	SHV-1; SHV-2[d]; SHV-5[d]; SHV-6; SHV-8[d]; SHV-19; SHV-20[d]; SHV-21[d]; SHV-22[d]; SHV-49; SHV-57
11v2	A (Gln) G C	*bla*$_{SHV-2a-11v2}$; *bla*$_{SHV-11-11v2}$; *bla*$_{SHV-12-11v2}$; *bla*$_{SHV-13-11v2}$; *bla*$_{SHV-15-11v2}$; *bla*$_{SHV-66-11v2}$; *bla*$_{SHV-70-11v2}$; *bla*$_{SHV-85-11v2}$	SHV-2a[d]; SHV-11; SHV-12[d]; SHV-13[d]; SHV-15[d]; SHV-66[d]; SHV-70[d]; SHV-86[d]
11v3	A (Gln) G G T G	*bla*$_{SHV-2a-11v3}$	SHV-2a[d]
11v4	A (Gln) T G	*bla*$_{SHV-2a-11v4}$	SHV-2a[d]
1v5	A G C G	*bla*$_{SHV-3-1v5}$	SHV-3[d]
1v6	G G	*bla*$_{SHV-5-1v6}$	SHV-5[d]
1v7	G A A G	*bla*$_{SHV-5-1v7}$	SHV-5[d]
1v8	T G A	*bla*$_{SHV-1-1v8}$; *bla*$_{SHV-7-1v8}$; *bla*$_{SHV-33-1v8}$	SHV-1; SHV-7[d]; SHV-33
1v9	C G C	*bla*$_{SHV-9-1v9}$	SHV-9[d]
1v10	T G A G	*bla*$_{SHV-14-1v10}$	SHV-14
1v11	T	*bla*$_{SHV-16-1v11}$	SHV-16
1v12	T G A G G	*bla*$_{SHV-1-1v12}$; *bla*$_{SHV-18-1v12}$; *bla*$_{SHV-30-1v12}$; *bla*$_{SHV-34-1v12}$; *bla*$_{SHV-38-1v12}$; *bla*$_{SHV-71-1v12}$; *bla*$_{SHV-72-1v12}$; *bla*$_{SHV-73-1v12}$; *bla*$_{SHV-83-1v12}$	SHV-1; SHV-18[d]; SHV-30[d]; SHV-34[d]; SHV-38; SHV-71; SHV-72; SHV-73; SHV-83
1v13	G	*bla*$_{SHV-24-1v13}$	SHV-24[d]
11v14	A (Gln) G A G	*bla*$_{SHV-25-11v14}$	SHV-25
1v15	G C G	*bla*$_{SHV-1-1v15}$; *bla*$_{SHV-26-1v15}$; *bla*$_{SHV-50-1v15}$; *bla*$_{SHV-60-1v15}$; *bla*$_{SHV-63-1v15}$; *bla*$_{SHV-78-1v15}$; *bla*$_{SHV-104-1v15}$	SHV-1; SHV-26; SHV-50; SHV-60; SHV-63; SHV-78; SHV-104[d]
1v16	T T G G	*bla*$_{SHV-27-1v16}$; *bla*$_{SHV-32-1v16}$	SHV-27; SHV-32
1v17	T T G	*bla*$_{SHV-27-1v17}$; *bla*$_{SHV-45-1v17}$	SHV-27; SHV-45
1v18	T G A A C	*bla*$_{SHV-1-1v18}$; *bla*$_{SHV-28-1v18}$; *bla*$_{SHV-55-1v18}$; *bla*$_{SHV-106-1v18}$	SHV-1; SHV-28; SHV-55[d]; SHV-106[d]
1v19	T G A A C G	*bla*$_{SHV-1-1v19}$; *bla*$_{SHV-28-1v19}$	SHV-1; SHV-28
1v20	T G T A A C	*bla*$_{SHV-28-1v20}$	SHV-28

Continued

Table 7.2 - Continued

Framework	Nucleotide nb. (aminoacid nb.): 51 57 92(35) 153 156 201 276 324 336 348 357 397 402 405 408 429 456 462 510 516 534 537 564 570 615 633 642 654 672 705 720 759 762 765 771 786 789 795 813 846	*bla*$_{SHV}$ gene	Encoded β-lactamase[b]
11v21	A (Gln) T G A G G	*bla*$_{SHV\text{-}29\text{-}11v21}$	SHV-29
11v22	A (Gln) G A C G	*bla*$_{SHV\text{-}31\text{-}11v22}$	SHV-31
11v23	A (Gln) T G A G	*bla*$_{SHV\text{-}35\text{-}11v23}$	SHV-35
11v24	A (Gln) T G C G	*bla*$_{SHV\text{-}11\text{-}11v24}$; *bla*$_{SHV\text{-}36\text{-}11v24}$; *bla*$_{SHV\text{-}80\text{-}11v24}$	SHV-11; SHV-36; SHV-80
11v25	A (Gln) G T A G	*bla*$_{SHV\text{-}37\text{-}11v25}$	SHV-37
1v26	T G A G A	*bla*$_{SHV\text{-}38\text{-}1v26}$	SHV-38
11v27	A (Gln) T G A C G	*bla*$_{SHV\text{-}11\text{-}11v27}$; *bla*$_{SHV\text{-}40\text{-}11v27}$; *bla*$_{SHV\text{-}69\text{-}11v27}$; *bla*$_{SHV\text{-}79\text{-}11v27}$; *bla*$_{SHV\text{-}85\text{-}11v27}$; *bla*$_{SHV\text{-}89\text{-}11v27}$	SHV-11; SHV-40; SHV-69[d]; SHV-79; SHV-85; SHV-89[d]
1v28	T G C G A	*bla*$_{SHV\text{-}41\text{-}1v28}$	SHV-41
1v29	C G A G	*bla*$_{SHV\text{-}42\text{-}1v29}$	SHV-42
1v30	T G A A A C	*bla*$_{SHV\text{-}43\text{-}1v30}$	SHV-43
1v31	A C G	*bla*$_{SHV\text{-}44\text{-}1v31}$	SHV-44
1v32	G A	*bla*$_{SHV\text{-}46\text{-}1v32}$	SHV-46[d]
1v33	A	*bla*$_{SHV\text{-}48\text{-}1v33}$; *bla*$_{SHV\text{-}102\text{-}1v33}$	SHV-48; SHV-102[d]
1v34	T T G A	*bla*$_{SHV\text{-}51\text{-}1v34}$	SHV-51
11v35	A (Gln) G c	*bla*$_{SHV\text{-}53\text{-}11v35}$	SHV-53
11v36	A (Gln) T G G	*bla*$_{SHV\text{-}56\text{-}11v36}$	SHV-56
1v37	C G A C G	*bla*$_{SHV\text{-}59\text{-}1v37}$	SHV-59
11v38	A (Gln) T G A	*bla*$_{SHV\text{-}61\text{-}11v38}$	SHV-61
11v39	A (Gln) T G A A	*bla*$_{SHV\text{-}11\text{-}11v39}$; *bla*$_{SHV\text{-}62\text{-}11v39}$	SHV-11; SHV-62
11v40	A (Gln) G T G	*bla*$_{SHV\text{-}64\text{-}11v40}$	SHV-64[d]
1v41	C G C G	*bla*$_{SHV\text{-}65\text{-}1v41}$	SHV-65[d]
11v42	A (Gln) T G	*bla*$_{SHV\text{-}11\text{-}11v42}$; *bla*$_{SHV\text{-}67\text{-}11v42}$	SHV-11; SHV-67[d]
11v43	A (Gln) G G G	*bla*$_{SHV\text{-}69\text{-}11v43}$	SHV-69[d]

Continued

Table 7.2 - Continued

Framework	Nucleotide nb. (aminoacid nb.): 51 57 92(35) 153 156 201 276 324 336 348 357 397 402 405 408 429 456 462 510 516 534 537 564 570 615 633 642 654 672 705 720 759 762 765 771 786 789 795 813 846	bla_{SHV} gene	Encoded β-lactamase[b]
11v44	A(Gln) T G C G	$bla_{SHV\text{-}74\text{-}11v44}$	SHV-74
1v45	T G A	$bla_{SHV\text{-}75\text{-}1v45}$	SHV-75
11v46	A(Gln) T G A C	$bla_{SHV\text{-}76\text{-}11v46}$	SHV-76
11v47	A(Gln) G A G T	$bla_{SHV\text{-}77\text{-}11v47}$	SHV-77
11v48	A(Gln) T T G	$bla_{SHV\text{-}11\text{-}11v48}$; $bla_{SHV\text{-}81\text{-}11v48}$	SHV-11; SHV-81
11v49	A(Gln) T G	$bla_{SHV\text{-}11\text{-}11v49}$; $bla_{SHV\text{-}82\text{-}11v49}$	SHV-11; SHV-82
11v50	A(Gln) T T G A A	$bla_{SHV\text{-}92\text{-}11v50}$	SHV-92
11v51	A(Gln) T T G T G	$bla_{SHV\text{-}93\text{-}11v51}$	SHV-93
11v52	A(Gln) T G T A G	$bla_{SHV\text{-}94\text{-}11v52}$; $bla_{SHV\text{-}96\text{-}11v52}$	SHV-94; SHV-96
1v53	G T A A G	$bla_{SHV\text{-}95\text{-}1v53}$	SHV-95
1v54	G T A G	$bla_{SHV\text{-}97\text{-}1v54}$	SHV-97[d]
1v55	G A A C A G	$bla_{SHV\text{-}101\text{-}1v55}$	SHV-101
1v56	T G	$bla_{SHV\text{-}103\text{-}1v56}$	SHV-103
1v57	T G C G	$bla_{SHV\text{-}107\text{-}1v57}$	SHV-107
1v58	T G A A	$bla_{SHV\text{-}108\text{-}1v58}$	SHV-108
1v59	T T G A C G	$bla_{SHV\text{-}1\text{-}1v59}$	SHV-1
1v60	A T G A G G	$bla_{SHV\text{-}1\text{-}1v60}$	SHV-1
1v61	G A C A G	$bla_{SHV\text{-}1\text{-}1v61}$	SHV-1
1v62 T	C G A C G	$bla_{SHV\text{-}1\text{-}1v62}$	SHV-1
1v63	G A G	$bla_{SHV\text{-}1\text{-}1v63}$	SHV-1
1v64	T G A C G	$bla_{SHV\text{-}1\text{-}1v64}$	SHV-1
1v65	T G A G G	$bla_{SHV\text{-}1\text{-}1v65}$	SHV-1
1v66	C G A A C G	$bla_{SHV\text{-}1\text{-}1v66}$	SHV-1
1v67	G A A C G	$bla_{SHV\text{-}1\text{-}1v67}$	SHV-1
1v68	C T T G A C G	$bla_{SHV\text{-}1\text{-}1v68}$	SHV-1

Continued

Table 7.2 - Continued

Framework	Nucleotide nb. (aminoacid nb.): 51	57	92 (35)	153	156	201	276	324	336	348	357	397	402	405	408	429	456	462	510	516	534	537	564	570	615	633	642	654	672	705	720	759	762	765	771	786	789	795	813	846	*bla*$_{SHV}$ gene	Encoded β-lactamase[b]
1v69								T					G																	A							G					*bla*$_{SHV-1-1v69}$	SHV-1
1v70																																				G					*bla*$_{SHV-1-1v70}$	SHV-1
11v71			A (Gln)					T					G																	A							G		T			*bla*$_{SHV-11-11v71}$	SHV-11
11v72			A (Gln)					T					G													A				A			C				G					*bla*$_{SHV-11-11v72}$	SHV-11
11v73			A (Gln)										G							T									A													*bla*$_{SHV-11-11v73}$	SHV-11
11v74			A (Gln)										G																			G										*bla*$_{SHV-11-11v74}$	SHV-11
11v75			A (Gln)										G																														*bla*$_{SHV-11-11v75}$	SHV-11
11v76			A (Gln)							T			G																														*bla*$_{SHV-11-11v76}$	SHV-11
11v77		T	A (Gln)								T		G																	A			C				G					*bla*$_{SHV-11-11v77}$	SHV-11
11v78			A (Gln)					T					G													A				A							G					*bla*$_{SHV-11-11v78}$	SHV-11
11v79			A (Gln)										G																	A		G					G					*bla*$_{SHV-11-11v79}$	SHV-11
11v80			A (Gln)								T		G																									G					*bla*$_{SHV-11-11v80}$	SHV-11
11v81			A (Gln)								T		G			T														A			C				G					*bla*$_{SHV-11-11v81}$	SHV-11
11v82			A (Gln)								T		G											C	A				A	C				A								*bla*$_{SHV-11-11v82}$	SHV-11
11v83			A (Gln)					T					G																									G					*bla*$_{SHV-11-11v83}$	SHV-11

[a]Known β-lactamase encoding genes with respective frameworks: 84 retrieved from NCBI and 215 sequenced in previous studies (unpublished results) (Mendonça *et al.*, 2006b).
[b]In respect to *bla*$_{SHV}$ genes .
[c]Parental gene, used as reference to define *bla*$_{SHV-1}$ and *bla*$_{SHV-11}$ variants.
[d]ESBL enzymes. The remaining are non-ESBL enzymes.

This codification is both flexible and open-ended, and allows naming of all frameworks of the *bla*$_{SHV}$ genes. The proposed classification may appear in the SHV enzyme designation (ex: *bla*$_{SHV-76-11v46}$ which encodes SHV-76-11v46).

A comparable system, based on *bla*$_{TEM}$ genes, proposed by Leflon-Guidout *et al.* (Leflon-Guibout *et al.*, 2000a) after that of Caniça *et al.* (Caniça *et al.*, 1997b), and currently used, as far as we are aware has no more than seven different gene sequence frameworks (from “a” to “g”), due to variants of nucleotides at seven positions (Leflon-Guibout *et al.*, 2000a; Pomba-Féria & Caniça, 2003). In a previous study Ford & Avison (2004), named different gene sequence frameworks “v1” to “v4” following the name of the *bla*$_{SHV}$ gene (ex: *bla*$_{SHV-1v4}$). We believe that our classification tends to be more complete.

When the gene sequence frameworks and the non-synonymous mutations other than T92A were considered, we observed that among the 297 *bla*$_{SHV}$ gene sequences 61% derived from *bla*$_{SHV-1}$ and 39% derived from *bla*$_{SHV-11}$ gene; the two most represented genes were *bla*$_{SHV-11}$ and *bla*$_{SHV-1}$ (each with 20%), with 20 and 18 gene sequence frameworks, respectively. Lee *et al* studied 142 *bla*$_{SHV}$ genes and reported 62% *bla*$_{SHV-11}$ genes and 35% *bla*$_{SHV-1}$ (Lee *et al.*, 2006b). The 297 *bla*$_{SHV}$ gene sequences comprised 135 different *bla*$_{SHV}$ genes encoding 36 ESBL and 99 non-ESBL enzymes. Among the ESBL encoding genes, we identified 22 (61%) with the *bla*$_{SHV-1}$ gene sequence framework and 14 (39%) with the *bla*$_{SHV-11}$ gene sequence framework. Among the non-ESBL encoding genes, 56 (57%) presented the *bla*$_{SHV-1}$ gene sequence framework and 43 (43%) the *bla*$_{SHV-11}$ gene sequence framework.

Here, we established a classification for the *bla*$_{SHV}$ gene family by taking into consideration the great variety of the gene sequence frameworks displayed by the available *bla*$_{SHV}$ genes. This study helps document the diversity of *bla*$_{SHV}$ genes, thereby contributing to a future understanding of the evolution of *bla*$_{SHV}$ family, and especially *bla* genes encoding ESBL enzymes.

CHAPTER VIII

CTX-M-15, OXA-30 and TEM-1-producing *Escherichia coli* in two Portuguese regions

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TEXT

Currently, CTX-M-type enzymes are the most common group of extended-spectrum beta-lactamases (ESBLs) not belonging to the TEM and SHV families (Livermore & Hawkey, 2005).

Here, we report the first phenotypic and molecular characterization of Portuguese *E. coli* strains harbouring CTX-M-15. Both strains were isolated in 2004. One strain (INSRA5753), isolated at Hospital Garcia de Orta in Lisbon, was from the urine of a 78-year-old woman who was living in a residential care home, using a catheter. The other strain (INSRA5754) was isolated from the blood of a 90-year-old woman 1 day after of hospitalization in Vila Real. These two hospitals are about 400 km apart. The strains were detected as ESBL-producers in the hospitals, using the VITEK 1 system and ATB G-5, respectively.

In the Antibiotic Resistance Unit, at the NIH in Lisbon, β-lactamase production was confirmed using the Etest ESBL screen method. Using PCR, fragments indicating the presence of bla_{TEM} gene, the bla_{OXA} gene, the bla_{CTX} gene and the ubiquitous *ampC* gene were amplified from both strains (Casin *et al.*, 1999; Edelstein *et al.*, 2003; Féria *et al.*, 2002). The amplification products from bla_{TEM}, bla_{OXA} and bla_{CTX} genes from both strains were purified and sequenced. For sequencing of the $bla_{CTX\text{-}M\text{-}15}$ gene, we used the primers specific for the consensus of CTX-M group I: CTX-M-15F, 5'-AGAATAAGGAATCCCATGGTT-3' and CTX-M-15R, 5'-ACCGTCGGTGACGA TTTTAG-3'.

Sequencing confirmed three resistance genes in both strains: the $bla_{TEM\text{-}1B}$, $bla_{OXA\text{-}30}$ and $bla_{CTX\text{-}M\text{-}15}$ genes. Sequence analysis using CTX-M-15 primers and IS*Ecp1*, IS*26* or IS*903r* primers (Eckert *et al.*, 2004) indicated the presence of an IS*Ecp1-like* element in both strains although neither had IS*903*, thus differing from mobile elements of $bla_{CTX\text{-}M\text{-}15}$ gene detected previously in *K. pneumoniae* (Conceição *et al.*, 2005). The IS*26* element was not also present in our strains. These findings suggest that CTX-M-15 enzymes in these two species in Portugal might have emerged in multiple places by plasmid acquisition of $bla_{CTX\text{-}M\text{-}15}$ genes, with different elements implicated in the dissemination of these β-lactamase genes (Conceição *et al.*, 2005). This was corroborated by the PFGE analysis, which showed different clonal origins for each isolate (data not shown).

The transconjugant C600-URA5753 was a CTX-M-15-positive-transconjugant from *E. coli* INSRA5753 derived using C600 as the recipient. The transconjugant C600-URA5754 co-expressed CTX-M-15, OXA-30 and TEM-1 enzymes; indeed, none of these enzymes were transferable alone from *E. coli* INSRA5754 strain to *E. coli* C600 (Table 8.1), suggesting that $bla_{CTX\text{-}M\text{-}15}$ gene was carried in only one transferable plasmid with $bla_{TEM\text{-}1B}$ and $bla_{OXA\text{-}30}$.

MICs of various β-lactams, alone or in combination with β-lactamase inhibitors (Table 8.1) were determined by an agar dilution method (Féria *et al.*, 2002), and MICs of other antimicrobial agents were determined by broth microdilution method. The clinical isolates and the transconjugants showed a higher level of resistance to cefotaxime, ceftriaxone, and aztreonam than to ceftazidime, which is characteristic of CTX-M producers. Isoelectric focusing revealed for both clinical strain and transconjugant C600-URA5754 produced β-lactamases with pIs of 5.4 (TEM-1), 7.5 (OXA-30) and 8.9 (CTX-M-15). Transconjugant C600-URA5753 produced a β-lactamase with a pI of 8.9.

Table 8.1 - MICs of β-lactam antibiotics for *E. coli* strains isolated in two hospitals, transconjugants, and recipients[a]

	MIC (mg/L) for *E. coli* strains:					
Antimicrobial drug	C600	K12-J53	C600-URA5753	INSRA5753	C600-URA5754	INSRA5754
Amoxicillin	4	>4,096	4,096	>4,096	4,096	>4,096
Amoxicillin + CLA[b]	≤2	64	16	128	32	256
Ticarcillin	8	>4,096	>4,096	>4,096	>4,096	>4,096
Piperacillin	0.5	256	>512	>512	512	>512
Piperacillin + TAZ[c]	0.5	2	32	8	2	32
Mecillinam	0.25	4	4	2	1	1
Cephalothin	4	32	>1,024	>1,024	>1,024	>1,024
Cefuroxime	2	4	>256	>256	>256	>256
Cefoxitin	2	16	4	8	2	16
Cefepime	0.125	0.06	64	32	8	64
Cefoperazone	≤0.25	4	>512	>512	256	>512
Ceftriaxone	0.03	0.06	>512	512	128	512
Ceftriaxone+ CLA	0.03	0.06	0.03	0.125	≤0.015	0.25
Cefotaxime	0.06	0.25	512	512	128	>512
Cefotaxime + CLA	0.03	0.06	0.03	0.125	≤0.015	0.25
Ceftazidime	0.125	0.125	128	64	32	64
Ceftazidime + CLA	0.06	0.125	0.25	0.5	0.125	0.5
Aztreonam	0.125	0.125	>256	128	128	256
Aztreonam + CLA	0.06	0.125	0.125	0.25	0.125	0.25
Imipenem	0.125	0.125	≤0.06	0.125	≤0.06	0.125
Ciprofloxacin	≤0.5	≤0.5	≤0.5	>2	≤0.5	>2
Tobramicin	≤2	≤2	≤2	>8	4	>8
Trimethoprim-Sulfamethoxazole	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19	≤1/19

[a] *E. coli* C600-URA5753 (harbouring CTX-M-15 enzyme) and *E. coli* C600-URA5754 (harbouring TEM-1B, OXA-30 and CTX-M-15 enzymes) were transconjugants of *E. coli* INSRA5753 (harbouring TEM-1B, OXA-30, CTX-M-15 enzymes) and INSRA5754 (harbouring TEM-1B, OXA-30, CTX-M-15 enzymes), respectively; *E. coli* C600 was the recipient; *E. coli* K12 J53 R111 (harbouring TEM-1 enzyme) was used as the control.
[b] CLA, clavulanic acid at a fixed concentration of 2 mg/L.
[c] TAZ, tazobactam at a fixed concentration of 4 mg/L.

To our knowledge the combination of CTX-M-15, TEM-1 and OXA-30 β-lactamases, here reported, was previously described only in strains from Senegal (Weill *et al.*, 2004). However, the combination of CTX-M-15 and OXA-1 (similar to OXA-30) as been described in the UK, Canada and India (Livermore & Hawkey, 2005; Boyd *et al.*, 2004). The spread of resistance to extended-spectrum cephalosporins, their extensive use, and the coexistence of

strains co-expressing the same TEM, OXA and CTX-M enzymes in distant regions of Portugal may impede the use of β-lactams in other regions of the country. These cases involving hospital and community environments are consistent with dissemination either from or to the community, which is of high concern.

CHAPTER IX

Spread of extended-spectrum β-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal

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ABSTRACT

Of the 181 unduplicated *E. coli* strains isolated in nine different hospitals in three Portuguese regions, 119 were ESBL-CTX-M producers and were selected for phenotype and genotype characterization. CTX-M producer strains were prevalent among community-acquired infections (56%), urinary tract infections (76%), and patients ≥ 60 years old (76%). In MIC tests, all strains were resistant to cefotaxime, 92% were resistant to ceftazidime, 93% were resistant to quinolones, 89% were resistant to aminoglycosides and 26% were resistant to trimethoprim-sulfamethoxazole; all strains were sensitive to carbapenems, and 92% had a multidrug resistance phenotype. Molecular methods identified 109 isolates harbouring a *bla*$_{\text{CTX-M-15}}$ gene, one harbouring the *bla*$_{\text{CTX-M-32}}$ gene (first identification in the country), and nine the *bla*$_{\text{CTX-M-14}}$ gene. All isolates presented the IS*Ecp1* element upstream from the *bla*$_{\text{CTX-M}}$ genes; one presented the IS*903* element (downstream of *bla*$_{\text{CTX-M-14}}$ gene) and none the IS*26* element; 85% carried *bla*$_{\text{TEM-1B}}$, and 84% also carried a *bla*$_{\text{OXA-30}}$. Genetic relatedness analysis based of pulsed-field gel electrophoresis defined five clusters, and indicated that 76% of all isolates (from cluster IV) corresponded to a single epidemic strain. Of the 47 strains from one hospital, 41 belonged to cluster IV, and were disseminated in three main wards. CTX-M-producing *E. coli* strains are currently a problem in Portugal, with CTX-M-15 particularly common. This study suggests that the horizontal transfer of *bla*$_{\text{CTX-M}}$ genes, mediated by plasmids and/or mobile elements, contributes to the dissemination of CTX-M enzymes to community and hospital environments. The use of extended-spectrum cephalosporins, quinolones and aminoglycosides is compromised, leaving carbapenems as the therapeutic option for severe infections caused by ESBL producers.

INTRODUCTION

Production of ESBLs is a major mechanism of resistance to β-lactam antibiotics. First detected in 1986 (Matsumoto *et al.*, 1988), CTX-M is an ESBL family including more than 60 different enzymes in five phylogenetic groups (http://www.lahey.org/studies/webt.htm). CTX-M β-lactamases of group 2be (Bush *et al.*, 1995), unlike other ESBL, are more active against cefotaxime than against ceftazidime (Bonnet, 2004). The rapid emergence and worldwide spread of this plasmid-mediated family is associated with mobile elements and particularly insertion sequences (Baraniak *et al.*, 2002a; Boyd *et al.*, 2004; Eckert *et al.*, 2006; Karim *et al.*, 2001; Munday *et al.*, 2004; Poirel *et al.*, 2005; Walther-Rasmussen & Høiby, 2004). This high mobility associated with inefficient antibiotic policies has led to community-acquired and nosocomial infections (Livermore & Hawkey, 2005; Woodford *et al.*, 2004).

Many strains that express CTX-M β-lactamases are multidrug resistant (Eisner *et al.*, 2006; Karim *et al.*, 2001; Livermore, 2005; Woodford *et al.*, 2004). Genes conferring

resistance to aminoglycosides and tetracycline and other *bla* genes have been found on the same plasmids as the bla_{CTX-M} genes (Boyd *et al.*, 2004). Genes conferring plasmid-mediated quinolone resistance has also been associated to bla_{CTX-M} genes (Jacoby *et al.*, 2006).

CTX-M-15 appears to have the best dissemination capacity of all the CTX-M family, probably due to successful genetic rearrangements (Walsh, 2006). The corresponding gene is normally associated with an upstream IS*Ecp1* element. The gene has been detected in Europe (Baraniak *et al.*, 2002a; Conceição *et al.*, 2005; Eckert *et al.*, 2004; Livermore and Hawkey, 2005), Africa (Moubareck *et al.*, 2005; Ndugulile *et al.*, 2005; Ramdani-Bouguessa *et al.*, 2006; Soge *et al.*, 2005), North and South America (Boyd *et al.*, 2004; Pallecchi *et al.*, 2004) and Asia (Karim *et al.*, 2001; Kim *et al.*, 2005), and has been associated with many community and hospital outbreaks. To our knowledge, only dispersed cases have been reported in Portugal (Conceição *et al.*, 2005; Costa *et al.*, 2004; Machado *et al.*, 2006; Machado *et al.*, 2004; Mendonça *et al.*, 2006b).

We investigate the dissemination of CTX-M enzymes among clinical isolates of *E. coli* recovered in various Portuguese hospitals from cases of community-acquired and nosocomial infections. We describe the phenotypes and genetic characteristics of these isolates.

MATERIALS AND METHODS

Bacterial strains. A total of 181 unduplicated *E. coli* strains isolated from patients, were collected in nine hospitals in three different regions of Portugal, between March 2004 and March 2006, and sent to the Antibiotic Resistance Unit at the NIH Dr. Ricardo Jorge in Lisbon. The bacteriology laboratories of these hospitals collaborate with the NIH as contributors to the ARSIP; they sent all *E. coli* strains identified as ESBL producers by different systems (ATB G-5, VITEK 1, VITEK 2 and Phoenix). Of the 181 strains received, 119 were detected as ESBL-(CTX-M)-producers by phenotypic and genotypic characterization at NIH, as stated below. The NIH conducted the main study using those 119 out of 126 CTX-M producer strains because only the first bacterial isolate from the three patients with multiple isolates was considered (among a total of 181 ESBL producers) (Table 9.1). The other ESBL producers were not investigated in present study. Of the 119 strains, 47 were nosocomial, and 66 were community acquired, according to Centers for Disease Control and Prevention criteria (Garner *et al.*, 1996), and six were of unknown origin (Table 9.1).

Table 9.1 - General characterization of the 119 CTX-M-producing *E. coli* strains studied (one per patient), characterized by hospitals, regions and origin of isolates

Hospital[a] code	Region	Origin and number of isolates						Total
		Community Acquired (n=66)		Nosocomial (n=47)		Unknown (n=6)		
		PFGE Profile (No. of strains)	Total	PFGE Profile (No. of strains)	Total (Ward)	PFGE Profile (No. of strains)	Total	
A	North	0009 (1), 0010 (7)	8	0009 (1)	1 (Internal Medicine)	-	-	9
B	South	0009 (1), 0010 (5) 0012 (1), 0013 (1)	8	0010 (2)	2 (Emergency, Internal Medicine)	-	-	10
C	North	0010 (27), 0003 (1)[c] 0005 (1),[c] 0011 (1) nd (1)[c]	31[b]	0010 (14), 0018 (1)[c] nd (1)[c]	16[b] (Cardiology, Surgery, Internal Medicine, Nephrology,[c] Pneumology,[c] ICU, Urology)	-	-	47
D	Lisbon and Tagus Valley	0002 (1),[c] 0010 (7)	8[d]	0007 (1), 0010 (7) 0016 (1)	9[d] (Cardiology, Surgery, Internal Medicine, Orthopedics, Pneumology, Gastroentorology, Observation)	0014 (1)[c]	1	18
E	Lisbon and Tagus Valley	0001 (1),[c] 0006 (1) 0010 (6)	8	0004 (1), 0006 (1), 0008 (1), 0010 (4), 0017 (1), 0019 (1)	9[e] (Emergency, Internal Medicine, Surgery, Nephrology, ICU, Paediatric, Neurology)	0006 (2) 0010 (1) 0019 (1)[f]	4	21
F	Lisbon and Tagus Valley	-	-	0010 (1)	1 (Infectious Diseases)	-	-	1
G	Lisbon and Tagus Valley	-	-	0010 (1)	1 (Internal Medicine)	-	-	1
H	Lisbon and Tagus Valley	-	-	0010 (5), 0015(1)	6 (Surgery, Internal Medicine)	0010 (1)	1	7
I	North	0003 (1),[c] 0010 (1) nd (1)	3	0010 (2)	2 (Internal Medicine)	-	-	5

[a] All hospitals were general hospitals.
[b] Two more strains were collected from the same patient: both were noted as community acquired, whereas the first was nosocomial (from Urology ward).
[c] CTX-M-14 producers.
[d] Three more strains were collected from the same patient (two from Internal Medicine and one from community).
[e] Two more strains were collected from the same patient, all noted as nosocomial (from the pediatric ward).
[f] CTX-M-32 producers. All other isolates were CTX-M-15 producers excepting those from CTX-M-14 producers.

Strains were isolated from urine (n= 90), wounds (n= 13), blood (n= 4), ascitic fluid (n= 3), sputum (n=3), bronchoalveolar lavage (n=2) and secretions (n=3) and gastric fluid (n=1). *E. coli* INSRA99 (IRT-2, pI 5.2), *E. coli* RP4 (TEM-2, pI 5.6) and *E. coli* SolRl 90 (AmpC, pI 9.2) were used only as control strains for IEF. *E. coli* R111 (TEM-1, pI 5.4; bla_{TEM-1} plus *ampC*), *S. enterica* serovar Typhimurium (OXA-1, pI 7.4; bla_{OXA-1}), *E. coli* C600 (SHV-1, pI 7.6; bla_{SHV-1}) and *E. coli* UA1526 (CTX-M-15, pI 8.9; $bla_{CTX-M-15}$) were used as control strains for both PCR and IEF. The control strains used for PCR with insertion sequence primers were: INSRA5753 (IS*Ecp1*), INSRA5776 (IS*903*) and Kp125 (IS*26*).

Susceptibility testing and ESBL confirmation. The MICs of 23 antibiotics were determined by a broth microdilution method (MicroScan Panel Sólo 1S) for all strains. The MICs of antibiotics were determined by the agar dilution method for all transformants obtained as previously (Mendonça *et al.*, 2006a). The results were interpreted using CLSI (formerly National Committee for Clinical Laboratory Standards) criteria (CLSI, 2007). Isolates were considered multidrug resistant if they had reduced susceptibility to three or more structurally unrelated antibiotics. ESBL production was confirmed by a broth microdilution method: strains with synergy between ceftazidime and ceftazidime-clavulanic acid were suspected of producing ESBL. To confirm ESBL producers, 52 of 119 strains were randomly chosen for further testing by the Etest ESBL method using strips with cefotaxime and ceftazidime both alone and associated with clavulanate; findings were interpreted according to the manufacturer's instructions.

Transfer of resistance. We tested whether the ESBL phenotypes of strains producing CTX-M-14, CTX-M-15 and CTX-M-32 enzymes were transferable. Plasmid DNA was extracted from six producer strains, representative of the different CTX-M-type enzymes detected, and used to transform *E. coli* DH5α by electroporation. Transformants were selected on Luria Broth medium containing 1 μg of cefotaxime/ml. PCR was used to test for the *bla* gene in transformants as described below for clinical isolates.

IEF. Cell extracts from all isolates were obtained by ultrasonic treatment and IEF was used to characterise pI of ESBLs as previously described (Caniça *et al.*, 1997a). The pIs of each β-lactamase were compared with those produced by control strains.

PCR amplification and gene sequencing. PCR amplification was used to test for the bla_{CTX-M} gene and IS*Ecp1*, IS*26* and IS*903* elements, and multiplex-PCR was used to test for the bla_{TEM}, bla_{OXA}, bla_{SHV}, and a*mpC* genes in all 119 ESBL producer strains as previously (Machado *et al.*, 2004; Mendonça *et al.*, 2006a; Pomba *et al.*, 2006). Specific primers were

used for PCR and sequencing (Table 9.2). A particular specific primer (CTX15i) was used to determine the nucleotides of codon 288, which distinguish *bla*$_{CTX-M-15}$ from *bla*$_{CTX-M-28}$. PCR products were purified and further sequenced as previously described (Mendonça *et al.*, 2006a).

Table 9.2 - Primers used for PCR amplification and sequencing

Gene	Primer[a]	Primer Sequence (5' → 3')	PCR product size (bp)	Reference or source
bla$_{TEM}$	P1	5'-TACGATACGGGAGGGCTTAC-3'	716	Belaaouaj *et al.*, 1994
	P2	5'-TTCCTGTTTTTGCTCACCCA-3'		
	FIN	5'-ATTCTTGAAGACGAAAGGGC-3'	1091	Caniça *et al.*, 1997b
	DEB	5'-ATGAGTAAACTTGGTCTGAC-3'		
	P3[b]	5'-TGGGTGAGCAAAAACAGGAA-3'		Caniça *et al.*, 1997b
	CLB[b]	5'-AATGAAGCCATACCAAACGA-3'		Belaaouaj *et al.*, 1994
bla$_{SHV}$	SHVf1	5'-TCAGCGAAAAACACCTTG-3'	471	M'Zali *et al.*, 1996
	SHVr2	5'-TCCCGCAGATAAATCACCA-3'		
bla$_{OXA}$	oxa1f	5'-TATCTACAGCAGCGCCAGTG-3'	199	Féria *et al.*, 2002
	oxa1r	5'-CGCATCAAATGCCATAAGTG-3'		
	oxa1sf	5'-ATGAAAAACACAATACATATC-3'	816	Casin *et al.*, 1999
	oxa1sr	5'-AATTTAGTGTGTTTAGAATGG-3'		
ampC	ampCf	5'-CCCCGCTTATAGAGCAACAA-3'	634	Féria *et al.*, 2002
	ampCr	5'-TCAATGGTCGACTTCACACC-3'		
bla$_{CTX-M}$	CTXf[c]	5'-TTTGCGATGTGCAGTACCAGTAA-3'	543	Edelstein *et al.*, 2003
	CTXr[d]	5'-CGATATCGTTGGTGGTGCCATA-3'		
bla$_{CTX-M-15}$	CTX15f	5'-AGAATAAGGAATCCCATGGTT-3'	875	This study
	CTX15r	5'-ACCGTCGGTGACGATTTTAG-3'		
	CTX15i[b]	5'-GGAATCTGACGCTGGGTAAA-3'		This study
bla$_{CTX-M-14}$	CTXg3f	5'-CTGATGTAACACGGATTGACC-3'	871	This study
	CTX14r	5'-CGATTTATTCAACAAAACCAG-3'		
IS*Ecp1*	ISEcp1	5'-AAAAATGATTGAAAGGTGGT-3'	Variable	Eckert *et al.*, 2004
IS*26*	IS26	5'-AGCGGTAAATCGTGGAGTGA-3'	Variable	Eckert *et al.*, 2004
IS*903*	IS903	5'-CGGTTGTAATCTGTTGTCCA-3'	Variable	Eckert *et al.*, 2004

[a] For PCR amplification and sequencing (except those in used only for internal sequencing).
[b] Only for internal sequencing.
[c] When used with primer ISEcp1, IS26, IS903, this also amplifies the downstream region of the *bla*$_{CTX-M}$ gene. Various sized PCR products expected.
[d] When used with primer ISEcp1, IS26, IS903, this also amplifies the upstream region of the *bla*$_{CTX-M}$ gene. Various sized PCR products expected.

PFGE. Of the 119 isolates, 116 were analysed by PFGE. Seven other strains from three patients with multiple isolates in different hospitals were also subjected to PFGE analysis (Table 9.1). Plugs were prepared from bacterial suspensions (300 µl) with an optical density

at 650 nm of 1.2 to 1.4 in phosphate buffer (50 mM). SeaPlaque GTG (Cambrex Bio Science Rockland, Rockland, ME) agarose was added (1.5% in 300 µl of TF buffer); the mixture was immediately taken up in a 1-ml syringe, and placed for exactly 4 min at -20ºC. The syringe was then incubated at room temperature for 10 min, and its end was cut off. The plugs were sliced into 1-mm-thick sections and incubated in EC buffer (6 mM Tris, 1 M NaCl, 0.1 M EDTA, 0.2% deoxycholic acid, 0.5% *N*-lauroylsarcosine and 0.5% Brij 58) for 3 hours at 37ºC. The EC buffer was replaced with ES lysis buffer (0.5 M EDTA and 1% *N*-lauroylsarcosine) plus proteinase K (1 mg/strain) and the samples incubated for 16 h at 55ºC. The plugs were then washed with distilled water for 5 min and with TE buffer (100 mM Tris and 10 mM EDTA) four times for 30 min each time. The DNA in the plugs was digested with 30 U of XbaI for 16 hours at 37ºC. PFGE was performed on a CHEF MAPPER PFGE apparatus (Bio-Rad, Hercules, CA) with 1.2% Seakem Gold agarose (Cambrex) in 0.5x Tris-borate-EDTA at 11ºC and 6 V/cm. The duration of the run was 24 h, with initial and final switch times of 0.1 s and 36 s, respectively. The gels were stained with ethidium bromide and photographed with Gel Doc 2000 (Bio-Rad). Banding patterns were analyzed by using BioNumerics software. The unweighted-pair-group method was used to construct a dendrogram based on PFGE XbaI restriction patterns of the 123 *E. coli* isolates. The Dice band-based similarity coefficient, with a band position tolerance of 1.0% and an optimization of 1.8%, was used for clustering. A cutoff value of 80% similarity was determined by the cluster cutoff method according to Bionumerics software. Isolates with a Dice band-based similarity coefficient value of >80% were considered to belong to the same cluster.

RESULTS

Clinical ESBL producer strains and antibiotic susceptibility. We confirmed that the 119 isolates included in the study were ESBL producers. The CTX-M-32 producer strain was from a wound in a 51 year-old man, and eight of the nine CTX-M-14 producers were isolated from urine, and one was from a wound. Most of CTX-M-15 producers (92%) were from urine from men or women ≥ 60 years old (data not shown).

A total of 90% of the isolates were resistant to 11 of the 14 β-lactam antibiotics tested: more than 98% CTX-M group 1 enzyme producers were resistant to ceftazidime and cefotaxime; all CTX-M group 9 producers were resistant to cefotaxime, and 11% resistant to ceftazidime (Table 9.3). Only 26% CTX-M group 1 producers showed nonsusceptibility to amoxicillin-clavulanate and 12% showed nonsusceptibility to piperacillin-tazobactam.

Table 9.3 - MIC_{50}, MIC_{90}, range, percentage of resistant and non-susceptible *E. coli* strains (n=119) producing enzymes, from CTX-M-1 and CTX-M-9 groups, collected in nine Portuguese hospitals

Antimicrobial Agent	MIC (µg/mL) for strains producing:									CLSI Breakpoints[b] (µg/ml)	
	CTX-M group 1[a] (n=110)					CTX-M group 9 (n=9)					
	MIC_{50}[c]	MIC_{90}[c]	Range	Susceptibility[e] R (%)[c]	Susceptibility[e] IR (%)[d]	MIC_{50}[c]	MIC_{90}[c]	Range	Susceptibility R (%)[c]	Susceptibility IR (%)[d]	S	R
Ampicillin	>16	>16	4 - >16	110 (100)	110 (100)	>16	>16	4 - >16	9 (100)	9 (100)	≤ 8	≥ 32
Ampicillin+Sulbactam	>16/8	>16/8	8/4 - >16/8	108 (98)	109 (99)	>16/8	>16/8	8/4 - >16/8	9 (100)	9 (100)	≤ 8/4	≥ 32/16
Amoxicillin+Clavulanic acid	8/4	16/8	4/2 - 16/8	3 (3)	28 (26)	≤ 4/2	8/4	≤ 4/2 - 16/8	0 (0)	0 (0)	≤ 8/4	≥ 32/16
Piperacillin	>64	>64	16 - >64	110 (100)	110 (100)	>64	>64	16 - >64	9 (100)	9 (100)	≤ 16	≥ 128
Piperacillin+Tazobactam	≤ 16	32	≤ 16 - 64	7 (6)	13 (12)	≤ 16	≤ 16	≤ 16 - 64	0 (0)	0 (0)	≤ 16/4	≥ 128/4
Ticarcillin	>64	>64	8 - >64	110 (100)	110 (100)	>64	>64	8 - >64	9 (100)	9 (100)	≤ 16	≥ 128
Cephalothin	>16	>16	8 - >16	110 (100)	110 (100)	>16	>16	8 - >16	9 (100)	9 (100)	≤ 8	≥ 32
Cefazolin	>16	>16	1 - >16	110 (100)	110 (100)	>16	>16	1 - >16	9 (100)	9 (100)	≤ 8	≥ 32
Cefuroxime	>16	>16	2 - >16	110 (100)	110 (100)	>16	>16	2 - >16	9 (100)	9 (100)	≤ 8	≥ 32
Cefotaxime	>32	>32	0.5 - >32	109 (99)	110 (100)	>32	>32	0.5 - >32	9 (100)	9 (100)	≤ 8	≥ 64
Ceftriaxone	>32	>32	2 - >32	110 (100)	110 (100)	>32	>32	2 - >32	9 (100)	9 (100)	≤ 8	≥ 64
Aztreonam	>16	>16	1 - >16	110 (100)	110 (100)	8	>16	1 - >16	1 (11)	2 (22)	≤ 8	≥ 32
Ceftazidime	>16	>16	0.5 - >16	108 (98)	109 (99)	2	>16	0.5 - >16	1 (11)	1 (11)	≤ 8	≥ 32
Cefepime	>16	>16	0.5 - >16	109 (99)	109 (99)	>16	>16	0.5 - >16	7 (78)	9 (100	≤ 8	≥ 32
Cefoxitin	≤ 4	8	≤ 4 - 16	1 (1)	5 (5)	≤ 4	16	≤ 4 - 16	0 (0)	1 (11)	≤ 8	≥ 32
Imipenem	≤ 0.5	≤ 0.5	≤ 0.5 - 8	0 (0)	0 (0)	≤ 0.5	≤ 0.5	≤ 0.5 - 8	0 (0)	0 (0)	≤ 4	≥ 16
Meropenem	≤ 1	≤ 1	≤ 1 – 8	0 (0)	0 (0)	≤ 1	≤ 1	≤ 1 – 8	0 (0)	0 (0)	≤ 4	≥ 16
Amikacin	≤ 8	16	≤ 8 - 32	4 (4)	8 (7)	≤ 8	16	≤ 8 - 32	0 (0)	0 (0)	≤ 16	≥ 64
Gentamicin	>8	>8	2 - >8	87 (79)	87 (79)	≤ 2	>8	≤ 2 - >8	2 (22)	2 (22)	≤ 4	≥ 16
Tobramycin	>8	>8	2 - >8	103 (94)	104 (95)	≤ 2	>8	≤ 2 - >8	2 (22)	2 (22)	≤ 4	≥ 16
Ciprofloxacin	>2	>2	0.5 - >2	108 (98)	108 (98)	≤ 0.5	>2	≤ 0.5 - >2	3 (33)	3 (33)	≤ 1	≥ 4
Norfloxacin	>8	>8	2 - >8	108 (98)	108 (98)	≤ 2	>8	≤ 2 - >8	3 (33)	3 (33)	≤ 4	≥ 16
Ofloxacin	>4	>4	0.5 - >4	108 (98)	108 (98)	≤ 0.5	>4	≤ 0.5 - >4	3 (33)	3 (33)	≤ 2	≥ 8
Trimethoprim+ Sulfamethoxazole	≤ 1/19	>2/38	≤ 1/19 - >2/38	28 (26)	28 (26)	≤1/19	>2/38	≤ 1/19 - >2/38	3 (33)	3 (33)	≤ 2/38	≥ 4/76

[a] There were a total of 99 CTX-M-15 producers and one CTX-M-32 producer, according to the phylogenetic group 1 defined by Bonnet (2004) (Bonnet, 2004).
[b] As scored according to CLSI guidelines: S, sensitive; R, resistant.
[c] MIC 50, MIC at which 50% of the isolates are inhibited.
[d] MIC90, MIC at which 90% of the isolates tested are inhibited.
[e] R, number of resistant isolates; IR, number of nonsusceptible isolates

Imipenem and meropenem were the only antibiotics effective against all isolates producing either group 1 or group 9 enzymes (Table 9.3). A total of 92% of the isolates were multidrug resistant: 50% were isolated in the community and 37% were isolated from hospitalized patients.

Transfer of antibiotic resistance. We tested whether *bla*$_{CTX-M}$ genes in six selected isolates were transferable by transformation of *E. coli* strain DH5α. Only three transformants were obtained: two representative of CTX-M group 1 isolates (one carrying the *bla*$_{CTX-M-15}$ plus *bla*$_{TEM-1B}$ genes and other carrying the *bla*$_{CTX-M-32}$ gene) and one representative of CTX-M group 9 isolates (carrying the *bla*$_{CTX-M-14}$ gene). Generally the transformants had antibiotic resistance profiles similar to those of their parental clinical isolates (Table 9.4). All clinical strains were resistant to ciprofloxacin, but none of the transformants maintained that condition. Transformants of *E. coli* strains harboring CTX-M-32 or CTX-M-14 enzymes, whose clinical strains were resistant to gentamicin and trimethoprim-sulfamethoxazole, presented susceptibility to those antibiotics.

IEF. As determined by IEF, 82% of the CTX-M group 1 strains (n=110) contained enzymes with pIs 5.4, 7.4 and 8.9 (Table 9.5), 9% contained enzymes of pIs 5.4 and 8.9; and 9% contained enzymes with pIs of 7.4 and 8.9. All CTX-M group 9 strains (n=9) contained an enzyme of pI 8.1 and 44% contained an enzyme with a pI of 5.4.

Identification of *bla* genes and IS elements. The PCR tests used detected a *bla*$_{CTX-M}$ gene and the *ampC* gene in all isolates. A total of 91 of the 119 strains (76%) contained also *bla*$_{TEM}$ and *bla*$_{OXA}$ genes, 13 (11%) contained *bla*$_{CTX-M}$ plus *bla*$_{TEM}$ but not *bla*$_{OXA}$ gene and 10 (8%) had *bla*$_{CTX-M}$ and *bla*$_{OXA}$ genes (Table 9.5). Sequencing identified the *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-32}$ genes in 109 (92%), nine (8%) and one (1%) strains, respectively; the *bla*$_{TEM-1B}$ gene in 101 (85%) strains, *bla*$_{TEM-1C}$ in one strain and *bla*$_{TEM-1A}$ in one strain (note that all these *bla*$_{TEM}$ genes had a P3 promoter region). One strain had more than one *bla*$_{TEM}$ gene sequence. A total of 101 (85%) strains harboured a *bla*$_{OXA-30}$ gene. No strain carried the *bla*$_{SHV}$ gene. In all strains, the IS*Ecp1* element was found upstream from the *bla*$_{CTX-M}$ genes. One strain also carried an IS*903* element downstream from its *bla*$_{CTX-M-14}$ gene (Table 9.5).

Clonality of CTX-M-producer strains. PFGE analysis was used to establish the genetic relatedness of the 116 CTX-M producer strains. We identified 19 PFGE profile types were identified: 14 included a single clone genetically unrelated to other isolates in the study (types 0001, 0002, 0004, 0005, 0007, 0008 and 0011 to 0018).

Table 9.4. MICs[1] of antibiotics for clinical isolates and *E. coli* transformants, and recipients[a]

Antimicrobial Agent[b]	DH5α	INSRA5776 (CTX-M-14 + TEM-1B)	DH5α-URA5776 (CTX-M-14)	INSRA5905 (CTX-M-15 + TEM-1B)	DH5α-URA5905 (CTX-M-15 + TEM-1B)	INSRA5924 (CTX-M-32 + TEM-1B)	DH5α-URA5924 (CTX-M-32)
Amoxicillin	8	>4096	>4096	>4096	>4096	>4096	>4096
Amoxicillin-Clavulanic acid*	8	32	32	64	32	128	8
Ticarcillin	4	>4096	>4096	>4096	>4096	>4096	>4096
Piperacillin	2	256	256	>512	512	>512	512
Piperacillin-Tazobactam†	1	8	2	2	2	4	2
Mecillinam	0.125	1	0.5	2	2	2	0.5
Cephalothin	8	>1024	1024	>1024	>1024	>1024	>1024
Cefuroxime	4	>256	>256	>256	>256	>256	>256
Cefoperazone	≤0.25	256	16	>512	256	256	64
Ceftriaxone	0.03	32	32	>512	256	256	64
Ceftriaxone-Clavulanic acid*	0.06	0.25	0.06	≤0.016	≤0.016	0.125	0.06
Cefotaxime	0.06	32	32	512	512	256	128
Cefotaxime-Clavulanic acid*	0.06	0.5	0.06	0.25	0.03	0.125	0.06
Ceftazidime	0.25	2	1	128	64	16	16
Ceftazidime-Clavulanic acid*	0.125	0.5	0.25	0.25	0.25	0.5	0.25
Aztreonam	0.06	8	8	128	64	64	64
Aztreonam-Clavulanic acid*	0.06	0.5	0.125	0.06	0.06	0.25	0.125
Cefepime	0.03	8	2	32	8	16	4
Cefoxitin	4	32	4	16	4	16	8
Imipenem	0.25	≤0.06	0.5	0.25	0.25	0.125	0.125
Ciprofloxacin	≤0.125	256	≤0.125	1024	≤0.125	8	≤0.125
Gentamicin	≤0.125	32	0.5	1	0.25	16	≤0.125
Trimethoprim	≤0.125	>64	≤0.125	0.25	≤0.125	64	≤0.125

[a] *E. coli* DH5α-URA5776 (harboring CTX-M-14 enzymes), *E. coli* DH5α-URA5905 (harbouring TEM-1B and CTX-M-15 enzymes), and *E. coli* DH5α-URA5924 (harbouring CTX-M-32 enzymes) were transformants of *E. coli* INSRA5776 (harbouring TEM-1B and CTX-M-14 enzymes), *E. coli* INSRA5905 (harbouring TEM-1B and CTX-M-15 enzymes) and *E. coli* INSRA5924 (harbouring TEM-1B and CTX-M-32 enzymes), respectively; *E. coli* DH5α was the recipient.

[b] *, Clavulanic acid, 2 μg/mL; †, tazobactam, 4 μg/mL.

Table 9.5 - Phenotypic and genotypic characteristics of 119 *E. coli* CTX-M producer strains

Antimicrobial resistance pattern[a]	pI(s)[b]	Genotype by PCR (Gene type by sequencing):[c]						PFGE Profile[d] (No. of strains)	Hospital code	Total No. of Strains
		bla$_{TEM}$	*bla*$_{CTX-M}$	*bla*$_{OXA}$	IS*Ecp1*	IS*26*	IS*903*			
AM A/S AT CF CT FX TX TZ XM KZ PM PI P/T TI CI NX OF AK GM TO	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0010	A	1
AM A/S A/C AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF GM TO	7.4;8.9	-	+ (15)	+ (30)	+	-	-	0010	D	2
AM A/S A/C AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF AK TO	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0006	E	1
AM A/S AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF AK GM TO	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0010	C	1
AM A/S AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF GM TO T/S	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0010	C	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF AK GM TO T/S	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0010	B	1
AM A/S AT CF CT TX TZ XM KZ PM PI P/T TI CI NX OF GM TO	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0010	C	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF GM TO T/S	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0009(1), 0010(13), 0012(1), 0013(1), 0015(1), 0017 (1), nd(1)	A, B, C, D, E, F, H	19
	5.4;7.4;8.9	+ (1)[e]	+ (15)	+ (30)	+	-	-	0010	C	1
	5.4;8.9	+ (1b)	+ (32)	-	+	-	-	0019	E	1
	5.4;8.9	+ (1b)	+ (15)	-	+	-	-	0019	E	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF GM TO	8.1	-	+ (14)	-	+	-	-	0005	C	1
	7.4;8.9	-	+ (15)	+ (30)	+	-	-	0007(1), 0010(3)	B, D, H	4
	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0016(1), 0009(2), 0010 (48), 0011(1)	A, B, C, D, E, G, H, I	52
	5.4;7.4;8.9	+ (1[a])	+ (15)	+ (30)	+	-	-	0010	H	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF TO T/S	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0010	I	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF GM	5.4;8.9[f]	+ (1b)	+ (15)	+ (30)	+	-	-	0010	A	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF TO	7.4;8.9	-	+ (15)	+ (30)	+	-	-	0010	D, E	3
	5.4;8.9	+ (1b)	+ (15)	-	+	-	-	0010	D	1
	5.4;7.4;8.9	+ (1b)	+ (15)	+ (30)	+	-	-	0006(2), 0008(1), 0010(7)	C, D, E, I	10
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF T/S	5.4;8.9	+ (1b)	+ (15)	-	+	-	-	0010	A	2
AM AT CF CT TX TZ XM KZ PM PI TI CI NX OF TO	7.4;8.9	-	+ (15)	+ (30)	+	-	-	0010	E	1
AM A/S AT CF CT TX TZ XM KZ PM PI TI CI NX OF[g]	5.4;8.9	+ (1b)	+ (15)	-	+	-	-	0006(1), 0010(1)	E	2
AM A/S CF CT TX XM KZ PI TI CI NX OF GM TO T/S	5.4;8.1	+ (1b)	+ (14)	-	+	-	+	0001	E	1
AM A/S CF CT TX XM KZ PI TI CI NX OF T/S	5.4;8.1	+ (1b)	+ (14)	-	+	-	-	nd	C	1
AM A/S AT CF CT TX XM KZ PM PI TI T/S[g]	5.4;8.9	+ (1b)	+ (15)	-	+	-	-	0004	E	1
AM A/S CF CT TX XM KZ PM PI TI T/S[g]	5.4;8.1	+ (1b)	+ (14)	-	+	-	-	0018	C	1
AM A/S CF CT TX XM KZ PM PI TI[g]	8.1	-	+ (14)	-	+	-	-	0002(1), 0003(2), nd(1)	C, D, I	4
	5.4;8.1	+ (1c)	+ (14)	-	+	-	-	0014	D	1
AM AT CF TX XM KZ PI TI[g]	5.4;8.9	+ (1b)	+ (15)	-	+	-	-	0010	E	1

[a] AM, Ampicillin; A/S, ampicillin-sulbactam; A/C, amoxicillin-clavulanic acid; AT, aztreonam; CF, cephalothin; FX, cefoxitin; CT, cefotaxime; TX, ceftriaxone; TZ, ceftazidime; XM, cefuroxime; KZ. Cefazolin; PM, cefepime; PI, piperacillin; P/T, piperacillin-tazobactam; TI, ticarcillin; CI, ciprofloxacin; NX, norfloxacin; OF, ofloxacin; AK, amikacin; GM, gentamicin; TO, tobramycin; T/S, trimethoprim-sulfamethoxazole.

[b] pIs were determined by IEF: 5.4 corresponds to TEM-1A, TEM-1B or TEM-1C enzyme production, 7.4 corresponds to OXA-30 production, 8.1 corresponds to CTX-M-14, and 8.9 corresponds to CTX-M-15 or CTX-M-32 enzyme production.

[c] *bla*$_{TEM}$ genes amplified by PCR were identified by sequencing as *bla*$_{TEM-1A}$, *bla*$_{TEM-1B}$ or *bla*$_{TEM\ -1C}$; *bla*$_{CTX-M}$ genes by sequencing were identified as *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ or *bla*$_{CTX-M\ -32}$; and *bla*$_{OXA}$ genes were identified as *bla*$_{OXA-30}$. +, Amplification by PCR; -, No amplification by PCR.

[d] PFGE profile and number of strains with each profile, deduced from the dendrogram presented on Figure 9.1. ND, not determined.

[e] More than one *bla*$_{TEM-1}$ present.

[f] β-Lactamase with pI 7.4, corresponding to OXA-30 enzyme production, was not detected by IEF.

[g] Non-multidrug-resistant pattern.

The five other types, defined as clusters I to V, included numerous related (with >80% similarity) or indistinguishable (100% homology) isolates (Figure 9.1). Of the 102 (88%) strains in these five clusters, 91 (89%) had profile type 0010 (cluster IV). These 91 isolates included clones from all hospitals (isolated from 53 outpatients, 36 inpatients and two with no information) (Tables 9.1 and 9.5, and Figure 9.1). Hospital C provided more isolates than any other hospital, including four single-isolate profile types (0005, 0011 and 0018) and two profile types including numerous isolates (0003 and 0010) (Figure 9.1).

All isolates from the internal medicine, attending, intensive care unit (ICU), and urology services of hospital C were profile type 0010. Only the nephrology, pediatric, and emergency services of hospital C presented clones belonging to other different profile types (Table 9.6).

Isolates of profile types 0006 to 0013, 0015 to 0017 and 0019, expressed a CTX-M enzyme of group 1, and were multidrug-resistant. Isolates of profile types 0001 and 0005 were also multidrug resistant and expressed CTX-M enzyme of group 9. Isolates with profile types 0002, 0003, 0014 and 0018 also produced the CTX-M group 9 enzyme but were not multidrug resistant (Table 9.5). Three patients (from three different hospitals, C, D and E) gave multiple isolates; all provided isolates with PFGE profile type 00010, although the patient from hospital D also gave one isolate of PFGE profile type 0007 (Table 9.1).

DISCUSSION

The prevalence of CTX-M-type β-lactamases has increased substantially since 1992 (Bonnet, 2004): in Portugal, they have been found in isolates from animals, healthy humans and patients (Costa *et al.*, 2004; Machado *et al.*, 2006). Here, we investigated the dissemination of these enzymes among clinical strains responsible for community and hospital-acquired infections.

In a survey conduced in 1999, no ESBL enzymes of the CTX-M family were detected in clinical *E. coli* isolates in Portugal (M. Caniça, unpublished data).

We included only those strains resistant to extended-spectrum cephalosporins and monobactam-producing CTX-M β-lactamases collected in three different regions of Portugal between March 2004 and March 2006: 119 (66%) CTX-M producer strains out of 181 ESBL producers. The majority of the strains had CTX-M enzymes of group 1, CTX-M-15 (n=109) and CTX-M-32 (n=1), and nine strains had CTX-M-14, an enzyme of group 9. Most of CTX-M-15 producers were isolated in urine from men or women ≥ 60 years old as in Spain (Oteo *et al.*, 2006).

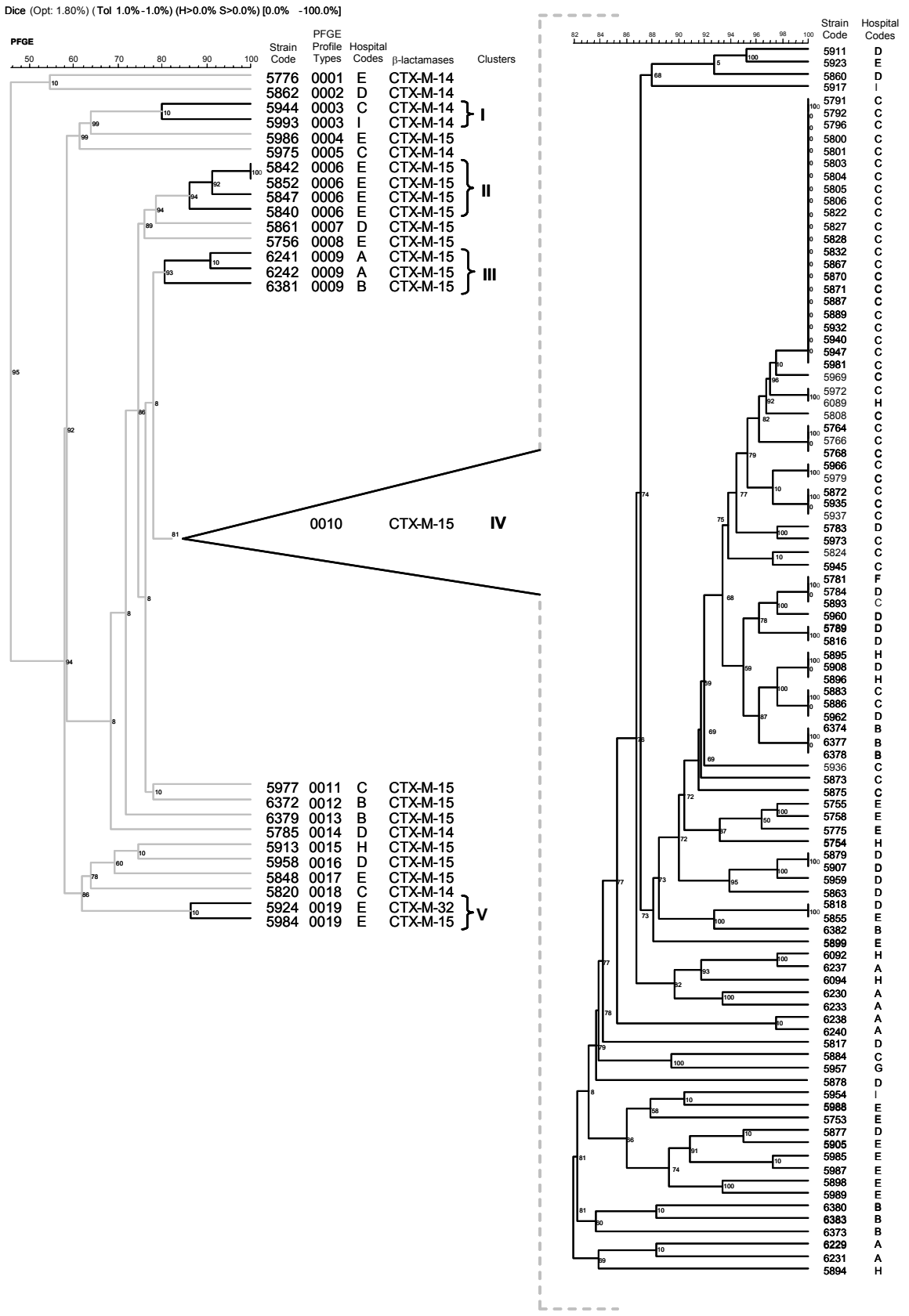

Figure 9.1 - Genetic relatedness among 123 *E. coli* strains by PFGE. PFGE profile types and clusters are shown from left to right. A total of 116 strains were the first isolate from each patient; seven additional isolates were collected from three of the patients. Strains INSRA5966, INSRA5979, INSRA5876, INSRA5879, INSRA5907, INSRA5863, INSRA5758 and INSRA5775 are multiple isolates of three patients. Strains with PFGE profile types 0003, 0006, 0009, 0010 and 0019 were defined as forming clusters I to V, respectively (indicated by vertical bands on the right).

Table 9.6 - Distribution of 47 strains from hospital C with corresponding PFGE profile types, by service, between May 2004 and May 2005

Service	Profile type (no. of strains)[a]												
	2004								2005				
	May	June	July	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Cardiology													0010(1)
Attending	0010(1)			0010(2)			0010(3)	0010(1)	0010(1)				
Emergency room							0010(1)		0010(1)	0010(2)	0011(1)	0010(1)	
Gastrology	0010(1)												
ICU										0010(1)			
Internal medicine	0010(1)	0010(2)	0010(1)	0010(3)	0010(1)	0010(4)	0010(2)	0010(1)	0010(1)				
Nephrology						0018(1)				ND			
Pediatric									0003(1)		0005(1)		
Pneumology			0010(1)						0010(ND)				
Surgery												0010(1)	
Unknown									0010(1)				
Urology							0010(2)	0010(3)					

[a] ND, not determined.

Strains producing CTX-M of group 1 were more resistant to ceftazidime and aztreonam (100%) than those producing CTX-M of group 9 (11%). This is consistent with previous reports (Bonnet, 2004). CTX-M-14 differs from its parental enzyme CTX-M-9 by a single substitution (Ala231Val), which does not confer an extension of resistance (Pai *et al.*, 2001; Sabaté *et al.*, 2000). CTX-M-32 differs from its parental enzyme CTX-M-1 by a single substitution (Asp240Gly), which confers high-level resistance to ceftazidime (Cartelle *et al.*, 2004). The same substitution is found in CTX-M-15 relative to its parental enzyme CTX-M-3, similarly conferring high-level resistance to ceftazidime (Poirel *et al.*, 2002b).

A total of 98% of strains producing CTX-M of group 1 were resistant to quinolones and 95% were resistant to aminoglycosides; only 33% and 22% of isolates producing CTX-M of group 9 were resistant, respectively. Resistance to quinolones (93%) was considerably more prevalent among our isolates than the 21% reported by Edelstein (Edelstein *et al.*, 2003); 89% of the isolates in both studies were resistant to aminoglycosides. Resistance to trimethoprim-sulfamethoxazole was more prevalent in isolates carrying CTX-M of group 9 (33%) than CTX-M of group 1 (26%).

Combined production of CTX-M and OXA enzymes by *E. coli* improved resistance to β-lactamase inhibitors. Less than 24% of the isolates were nonsusceptible to clavulanate and tazobactam, and more than 98% were resistant to sulbactam (Table 9.3). The usefulness of β-lactams plus a β-lactamase inhibitor is uncertain for patients with treatment failure (Livermore & Hawkey, 2005). Ninety-two percent of our strains harboring $bla_{\text{CTX-M-15}}$ gene (Table 9.5) also possessed a $bla_{\text{OXA-30}}$ gene, presumably explaining the high percentage of nonsusceptibility to ampicillin-sulbactam. Strains carrying $bla_{\text{CTX-M}}$ of group I enzymes were the only strains not susceptible to amoxicillin-clavulanate and to piperacillin-tazobactam which may imply a low expression of β-lactamase OXA-30 or even another mechanism of resistance (Oliver *et al.*, 1999).

The genes $bla_{\text{CTX-M-15}}$ plus $bla_{\text{OXA-1}}$ ($bla_{\text{OXA-30}}$) have been found associated in the same strain in India (Karim *et al.*, 2001), the UK (Livermore & Hawkey, 2005) and Canada (Boyd *et al.*, 2004). Note that $bla_{\text{OXA-1}}$ and $bla_{\text{OXA-30}}$ have the same sequence, and thus code for the same enzyme, called OXA-1 or OXA-30 (Ouellette *et al.*, 1987; Siu *et al.*, 2000). The combination of $bla_{\text{CTX-M-15}}$ plus $bla_{\text{OXA-30}}$ (or $bla_{\text{OXA-1}}$) plus $bla_{\text{TEM-1}}$ has been reported in seven strains from Korea (Jacoby *et al.*, 2006), in two strains from Senegal (Weill *et al.*, 2004) and also in Spain (Oteo *et al.*, 2006). An association between $bla_{\text{CTX-M-14}}$ and $bla_{\text{TEM-1B}}$ has been described in Korea (Kim *et al.*, 2005). However, our detection of $bla_{\text{CTX-M-32}}$ in the same strain as $bla_{\text{TEM-1B}}$ is, to our knowledge, a first.

There was an IS*Ecp1* element upstream from all $bla_{\text{CTX-M}}$ genes detected. Thus, we add the $bla_{\text{CTX-M-32}}$ gene to the list of $bla_{\text{CTX-M}}$ genes associated with the IS*Ecp1* element (Baraniak *et al.*, 2002a; Boyd *et al.*, 2004; Eckert *et al.*, 2006; Karim *et al.*, 2001; Munday *et*

al., 2004; Poirel *et al.*, 2005; Walther-Rasmussen & Høiby, 2004), which contributes to their mobilization (Poirel *et al.*, 2005). The IS*903* element was detected downstream from only the $bla_{CTX\text{-}M\text{-}14}$ gene, a finding in agreement with other studies (Eckert *et al.*, 2006; Machado *et al.*, 2006). The IS*26* element, characteristic of the epidemic strain A from the United Kingdom, was not detected among our strains, but was recently detected in clinical isolates from Spain that were collected in the same period as the strains of our study (Oteo *et al.*, 2006; Woodford *et al.*, 2004).

We tested for gene transfer from six isolates and genes were successfully transferred by electroporation to another *E. coli* strain from only three. This may suggest a major horizontal transfer by mobile elements. We obtained transformants containing CTX-14 alone, CTX-M-32 alone, and CTX-M-15 plus TEM-1B. The clinical strain containing CTX-M-14 enzyme was the only one not susceptible to cefoxitin; however, the transformant was susceptible indicating another mechanism of resistance. None of the transformants presented the same resistance to quinolone, aminoglycosides and trimethoprim as the clinical strains. Nevertheless, plasmid-determined resistance to quinolones and aminoglycosides, recently described in Portugal (Machado *et al.*, 2006), involving a variant of aminoglycoside acetyltransferase AAC(6')-Ib (Robicsek *et al.*, 2006), was not possible to identify in the three studied strains.

Resistance to quinolones (93%), aminoglycosides (89%) and trimethoprim-sulfamethoxazole (26%) explains why most CTX-M producers were multidrug resistant. Another study reported lower prevalences of non-susceptibility to quinolones (55%) and aminoglycosides (37%), but more frequent resistance to trimethoprim-sulfamethoxazole (34%) (Eisner *et al.*, 2006), and yet another describes very different percentages of resistance for trimethoprim-sulfamethoxazole (78% resistance) and aminoglycosides (43%) (Moubareck *et al.*, 2005). Eckert *et al* (2004) reported that 58% of isolates were resistant to trimethoprim-sulfamethoxazole, 74% to aminoglycosides, and 100% of CTX-M enzymes producers were multidrug resistant. In our study, the value was 92% for multidrug resistance, with a higher percentage of isolates from community (50%) than from hospital environments (37%), which was consistent with predominance of *E. coli* expression CTX-M enzyme in the community described by Pitout *et al* (Pitout *et al*., 2005). Indeed, the high-level consumption of antibiotics in outpatients in Portugal may be responsible for this resistance (Goossens *et al*., 2005). A wide diversity of resistance genes of numerous families coding for various antibiotic resistance mechanisms have now been described in CTX-M-15 producer strains.

We used PFGE to classify the strains. Three quarters of the strains clustered together (>80% similarity) in cluster IV, indicating countrywide dissemination of this multidrug resistant clone. Various factors may have contributed to this dissemination: the small size of the country, the proximity of population areas, and/or an inadequate antibiotic use. Since all

strains were susceptibility to carbapenems, the use of these or other appropriate drugs could help reduce the prevalence of strains from cluster IV.

Note that similar values for susceptibility to carbapenems were reported by others (Livermore, 2005; Soge *et al.*, 2005; Weill *et al.*, 2004). The spread of a single clone in hospital C suggested nosocomial dissemination, especially in internal medicine service. Nevertheless, the genetic similarity of clones in community services, in particular the attending and emergency rooms, also suggest dissemination within the community. The hospital-community and community-hospital dissemination of $bla_{\text{CTX-M-15}}$ enzyme, mainly in hospital C (Table 9.1 and 9.6), suggest the presence of an epidemic strain. The analysis of the three patients with multiple isolates shows that nosocomial infections due to *E. coli* CTX-M-15 producer strains are persistent (hospital D and E); that nosocomial infection is easily transferred among services (hospital D); and that nosocomial infection can become a community infection (hospital C and hospital D) (data not shown).

Our study suggests that *E. coli* CTX-M-producers are widespread in at least three regions of Portugal, possibly a consequence of the dissemination of major clones between hospitals and community and between regions and of the horizontal transfer of plasmids or mobile elements. Our findings also illustrate the potential dangers that the misuse of antibiotic can cause and the importance of measures to control infection. Nevertheless, rational use of other drugs may improve the situation. As previously noted (Livermore & Hawkey, 2005), the use of extended-spectrum cephalosporins, quinolones and aminoglycosides could be replaced with carbapenems for in infections in which ESBL-producing strains are likely to emerge.

CHAPTER X

CTX-M-3 and CTX-M-15 extended-spectrum β-lactamases in isolates of *Escherichia coli* from a hospital in Algiers, Algeria

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ABSTRACT

Sixteen strains of *E. coli* isolated between January and June 2005 in a hospital in Algiers carry the IS*Ecp1* element and TEM and either CTX-M-3 (n=3) or CTX-M-15 (n=13) β-lactamases. Fourteen of the isolates are multidrug resistant. Five isolates from the neonatal ward were indistinguishable by pulsed-field gel electrophoresis.

TEXT

CTX-M-type enzymes are the ESBL most commonly produced by *Enterobacteriaceae* (Bonnet, 2004), and more than 55 CTX-M-type β-lactamases have been described (http://www.lahey.org/studies/webt.htm). Despite the prevalence of ESBL in *Enterobacteriaceae*, data from Algeria are scarce (although the prevalence has been reported to be 20 to 45%) (Ramdani-Bouguessa *et al.*, 2001). We investigated the phenotypic and genetic profiles of clinical *E. coli* ESBL producers isolated in an Algerian hospital. (This work was presented at the 16th European Congress of Clinical Microbiology and Infectious Diseases, abstract P509, 2006).

Between January and June 2005, 279 non-duplicate *E. coli* strains were recovered consecutively from patients at the Mustapha Pacha Hospital (1,800 beds) of Algiers, Algeria, and routinely analyzed in the hospital's microbiology laboratory. All strains were identified with an API 20E System. Antimicrobial susceptibility was determined by disc diffusion according to the NCCLS guidelines (2004), and 22 (7.9%) of the strains were resistant to extended-spectrum cephalosporins. Only 16 of these 22 were available for this study; related specimens, patient age, and ward of hospitalization are specified in Table 10.1. A double disc diffusion test (Jarlier, 1988) and Etest ESBL strips, with cefotaxime and ceftazidime plus clavulanate, confirmed that all were ESBL producers.

All 16 isolates were positive for bla_{TEM}- and bla_{CTX-M}-related genes, and negative for bla_{OXA} and bla_{SHV} genes as assessed by PCR using previously described specific primers (Mendonça *et al.*, 2006a), and all isolates carried the ubiquitous *ampC* gene (Mendonça *et al.*, 2006a). Isoelectric focusing confirmed that all strains expressed both TEM-derived (pI of 5.4) and CTX-M-derived (pI of 8.0 and 8.9) enzymes (Table 10.1). The presence of an IS*Ecp1* element upstream from bla_{CTX-M} genes and the absence of IS*26* and IS*903* elements were shown by PCR experiments (Eckert *et al.*, 2004). ExoSAP IT was used for purification of PCR products, which were sequenced with an automatic sequencer ABI3100. Thirteen isolates carried the $bla_{CTX-M-15}$ gene and three the $bla_{CTX-M-3}$ gene; 16 isolates carried a $bla_{TEM-1B-type}$ gene.

MICs of antibiotics were determined by broth microdilution (MicroScan Panel Sólo 1S): 100% of strains were resistant to gentamicin, 31% to amikacin, 88% to cotrimoxazole,

and 19% to ciprofloxacin; 88% were multidrug-resistant (Table 10.1). The 13 isolates carrying both TEM and CTX-M-15 enzymes were more resistant to ceftazidime (with MICs >16 µl/ml), than the 3 CTX-M-3 plus-TEM producers (with MIC ≤0.5 to 1 µg/ml). CTX-M-15, which harbors the Asp240Gly substitution, confers higher levels of resistance to ceftazidime than its parental enzyme CTX-M-3 (Poirel *et al.*, 2002b).

The diversity of the isolates was investigated by a protocol for PFGE modified from that previously described (Caniça *et al.*, 2003), using *XbaI*-digested genomic DNA as suggested for *E. coli* (Davis *et al.*, 2003) (Figure 10.1). PFGE was performed on a CHEF MAPPER PFGE apparatus using a run time of 24 h, with an initial and final switch time of 0.1 s and 36 s, respectively. Strain INSRA5754 and the Lambda ladder (Biolabs, Beverly, MA) were used as markers for intragel normalization and intergel comparison. The PFGE profiles of five isolates producing CTX-M-15 β-lactamase from the neonatal ward were indistinguishable (100% similarity, cluster I). This suggests the spread of an epidemic clone. Two other clones producing CTX-M-3 β-lactamase were closely related (with >90% similarity, formed the cluster II). The PFGE profiles of the other isolates were heterogeneous.

ESBL-positive *Enterobacteriaceae* are frequently isolated in hospitals in Algeria, and the overall frequency of ESBL producers at the Mustapha Pacha hospital from January to June 2005, was 20.4% (n=217 of the 1,066 *Enterobacteriaceae* isolates): 22 of 279 (7.9%) *E. coli* isolates, 131 of 259 (50.6%) *Klebsiella sp* isolates, eight of 131 (6.1%) *Proteus sp* isolates, 35 of 90 (38.9%) *Enterobacter sp* isolates, 13 of 48 (27.1%) *Serratia sp* isolates, two of 19 (10.5%) *Morganella morganii* isolates, four of 18 (22.2%) *Citrobacter sp* isolates, and two of 14 (14.3%) *Salmonella sp*. isolates CTX-M-15 has been described in Asia, Europe and recently in Africa (Baraniak *et al.*, 2002a; Edelstein *et al.*, 2003; Gangoue-Pieboji *et al.*, 2005; Karim *et al.*, 2001; Soge *et al.*, 2006), both in nosocomial and community-acquired *E. coli* isolates (Moubareck *et al.*, 2005; Woodford *et al.*, 2004). Several studies in African countries report a high prevalence of ESBL-producing *Enterobacteriaceae* (Blomberg *et al.*, 2005; Gangoue-Pieboji *et al.*, 2005; Ramdani-Bouguessa *et al*., 2001; Soge *et al.*, 2006). There have been reports of ESBL producers in North Africa: TEM-3 in *S. enterica* serovar *typhymurium* in Morocco (AitMhand *et al*., 2002), CTX-M-27 in *S. enterica* serovar *livingstone* in Tunisia (Bouallègue-Godet *et al.*, 2005), and CTX-M-3 in *S. enterica* serovar *senftenberg* in Algeria (Naas *et al.*, 2005).

Table 10.1 - Distribution, clinical features, and phenotypic and genotypic characteristics of 16 ESBL-producing *E. coli* strains[a]

Strains	Patient Age	Specimen source	Hospital ward	pIs	β-lactamases produced	MICs (µg/mL) of[b]:							Other resistance marker(s)
						AMC	AZT	CTX	CAZ	CAZ/CA	FOX	IMP	
50	23 years	Wound	Orthopedic surgery	5.4, 8.0	TEM-1, AmpC, CTX-M-3	≤4/2	16	>32	1	≤0.5	≤4	≤0.5	GM, TOB, AN, CHL, SXT
53	47 years	Ascitic fluid	Gastroenterology	5.4, 8.9	TEM-1, AmpC, CTX-M-15	8/4	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, AN, OFX, SXT
95	4 years	Urine	Pediatric	5.4, 8.0	TEM-1, AmpC, CTX-M-3	≤4/2	16	>32	≤0.5	≤0.5	≤4	≤0.5	GM, TOB, AN, SXT
97	32 years	Wound	Digestive surgery	5.4, 8.0	TEM-1, AmpC, CTX-M-3	8/4	>16	>32	1	≤0.5	≤4	≤0.5	GM, TOB, SXT
102	3 years	Urine	Pediatric	5.4, 8.9	TEM-1, AmpC, CTX-M-15	>16/8	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, AN, CHL, SXT
108	20 days	Urine	Pediatric	5.4, 8.9	TEM-1, AmpC, CTX-M-15	≤4/2	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, SXT
109	24 years	Wound	Gastroenterology	5.4, 8.9	TEM-1, AmpC, CTX-M-15	≤4/2	>16	>32	16	≤0.5	≤4	≤0.5	GM, TOB
131	2 days	CSF	Neonatal	5.4, 8.9	TEM-1, AmpC, CTX-M-15	8/4	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, SXT
143	86 years	Urine	Outpatient	5.4, 8.9	TEM-1, AmpC, CTX-M-15	8/4	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, AN, CHL, OFX, SXT
163	8 days	Blood	Neonatal	5.4, 8.9	TEM-1, AmpC, CTX-M-15	8/4	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, SXT
165	12 years	Urine	Pediatric	5.4, 8.9	TEM-1, AmpC, CTX-M-15	>16/8	>16	>32	>16	>2	>16	≤0.5	GM, TOB, SXT
168	14 years	Blood	Pediatric	5.4, 8.9	TEM-1, AmpC, CTX-M-15	≤ 4/2	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, SXT
171	16 days	CSF	Neonatal	5.4, 8.9	TEM-1, AmpC, CTX-M-15	≤ 4/2	>16	>32	>16	≤0.5	≤4	≤0.5	GM, SXT
192	9 years	Blood	Pediatric	5.4, 8.9	TEM-1, AmpC, CTX-M-15	≤ 4/2	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, SXT
229	3 days	Blood	Neonatal	5.4, 8.9	TEM-1, AmpC, CTX-M-15	≤ 4/2	>16	>32	>16	≤0.5	≤4	≤0.5	GM
254	8 years	Wound	Pediatric surgery	5.4, 8.9	TEM-1, AmpC, CTX-M-15	≤ 4/2	>16	>32	>16	≤0.5	≤4	≤0.5	GM, TOB, AN, OFX, SXT

[a] CSF, cerebrospinal fluid; AMC, Amoxicillin-clavulanic acid; AZT, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; CAZ/CA, ceftazidime-clavulanic acid;FOX, cefoxitin; IMP, imipenem; GM, gentamicin; TOB, tobramycin; AN, amikacin; CHL, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; and OFX, ofloxacin.
[b] When two values separated by a slash are given for the MIC, the first value is of the antibiotic alone and the second value is of the antibiotic in the presence of clavulanate.

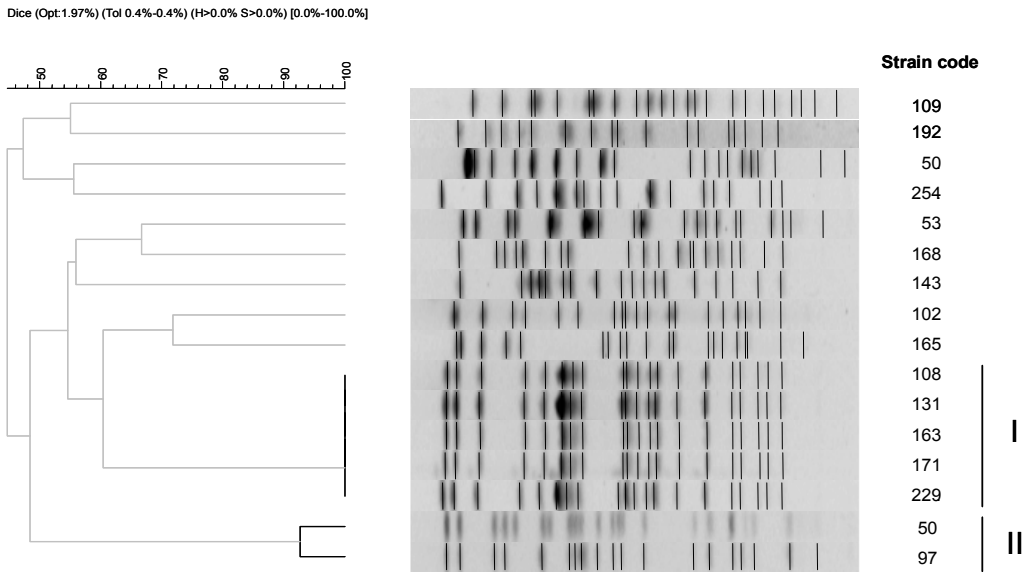

Figure 10.1 - Genetic relatedness of the 16 *E. coli* strains as assessed by PFGE. A band position tolerance of 0.4% was used in PFGE pattern analysis with the Dice band-based similarity coefficient. Strain code numbers are shown on the right. Clones in clusters I and II (indicated by vertical bands on the right) contain isolates of >80% similarity. Clones in cluster I were 100% identical and those in cluster II had >90% similarity

The frequency of *Enterobacteriaceae* producing ESBL in Algeria has not been reported.

The production of similar TEM and CTX-M-type enzymes in various genetically related strains and in isolates from different wards of the hospital suggests horizontal transfer of the corresponding genes. Five CTX-M-15-producing isolates were genetically indistinguishable; they were isolated from patients in the neonatal ward, except for isolate 108 which was from a patient hospitalized elsewhere for 20 days but who had previously been in this ward. Three of the patients in the neonatal ward were preterm (with 31 to 34 weeks): the patient infected with strain 171 had nosocomial meningitis, and the patients with isolates 131 and 229 had probably acquired the infection by transmission from the mother. The two cases of meningitis were cured but the case of bacteremia was fatal.

Invasive infections due to *E. coli* producing ESBL are a major problem in neonates, because the choice of drug is restricted. The widespread use of cefotaxime and ceftriaxone has been suggested to be have favored the emergence of CTX-M-enzymes (Wang *et al*., 2003). However, treatment of infections with ESBL-producing strains in this hospital usually do not involves those antibiotics for meningitis. Therefore, this hospital may have experienced the spread of an epidemic clone not directly due to antibiotic selection pressure, but with ISE*cp1* insertion sequences, involved in the mobilization of CTX-M-enzymes,

contributing to the process. Dissemination of community clones in the hospital environment is also a possibility.

To our knowledge this is the first report of CTX-M enzymes in *E. coli* from Algeria. We show that CTX-M-15 is widespread among *E. coli* isolates which are multidrug resistant, substantially restricting therapeutic alternatives. Implementation of a strict hospital infection control policy associated with efforts to promote judicious use of antibiotics is needed. Continuous monitoring of ESBL-producing *Enterobacteriaceae* in the community and the hospital setting is also required.

CHAPTER XI

Concluding Remarks

Antimicrobial resistance to antibiotics is a growing problem worldwide. ESBL-producing organisms remain an important cause of cephalosporin therapy failure with serious consequences for infection control (Paterson & Bonomo, 2005). The results presented in this dissertation answer several important questions, such as how is the CTX-M family disseminated in clinical isolates of *E. coli* in community and nosocomial environments? Or which specific amino acid substitutions promote the resistance to β-lactamase inhibitors or improve the resistance to extended-spectrum cephalosporins and what is their function within the resistance mechanism? On the other hand, this work raised other as of yet unanswered questions.

In the end of chapters II to X, which consist of published or submitted publications (chapters II to VII on *K. pneumoniae*, chapters VIII to X on *E. coli*), the results presented in each one of the publications are discussed. However, an overall discussion of the different chapters is valuable.

By far the most important mechanism of resistance to β-lactam antibiotics, the β-lactamases, could confer loss of susceptibility to several antibiotic classes like penicillins, narrow-, extended-, and broad-spectrum cephalosporins and also to β-lactam/β-lactamase inhibitor combinations. ESBL-producing *Klebsiella* spp and *E. coli*, have been reported as one of the six drug-resistant microbes to which new therapies are urgently needed (Talbot *et al.*, 2006).

The rapidly evolving ESBL enzymes are inhibited by clavulanic acid, although the emergence of CMT compromises this inhibition. These enzymes are frequently encoded in plasmids that may simultaneously possess other genes responsible for resistance to different antibiotic classes, like quinolones and aminoglycosides, which is a further limiting factor to viable treatment options. Furthermore, ESBL-producing organisms may often present a non-ESBL phenotype, by being susceptible to extended-spectrum cephalosporins, which could increase failure rates of treatment options (Lin *et al.*, 2006). Thus, the presence of ESBLs carries tremendous clinical significance. It has long been documented that the cephalosporins breakpoints presently used fail to detect many of ESBLs in *Enterobacteriaceae*.

Therefore, in Chapter II, CLSI guidelines were compared with reference laboratory criteria, that classify as ESBL producer strains for which any MIC of extended-spectrum cephalosporins is 3-fold lower in the presence of an established concentration of clavulanate. We obtained lower values of sensitivity for the agar dilution and broth microdilution methods when interpreted using CLSI guidelines in 2006, which proves that breakpoints should be lowered. In fact, recent breakpoint revision from CLSI guidelines, reduce the nonsusceptible MIC breakpoint to ≥ 2 mg/L for cefotaxime and ≥ 8 mg/L for ceftazidime instead of ≥ 16 mg/L previously established for both antibiotics (Kahlmeter, 2008). This revision agrees with the

MIC breakpoints of the newly established EUCAST guidelines, although for ceftazidime MIC breakpoint has been established even lower (≥ 2 mg/L). These new breakpoints would increase sensitivity for both techniques, thus improving results of susceptibility tests, and obviating the use of genotypic methods as ancillary tests.

A major issue that concerned both EUCAST and CLSI committees was if the new *Enterobacteriaceae* MIC breakpoints would be able to predict clinical success or failure, by correctly identifying ESBL producers without the need of complementary methods. Based on the available data, and besides lowering the existing breakpoints, both committees concluded that detection and characterisation of new ESBLs would continue to be important for infection control and surveillance purposes (Kahlmeter, 2008).

The detection and identification of a new ESBL, SHV-55 (Chapter II), allowed for the biochemical characterization of this enzyme (Chapter III). Although when using MIC evaluation tests SHV-55 conferred a higher level of resistance to ceftazidime than to cefotaxime, kinetic studies showed higher affinity, enzymatic activity and catalytic efficiency towards cefotaxime than for ceftazidime. Similar results had been previously obtained for SHV-5 (Gutmann *et al.*, 1989; Mulgrave & Attwood, 1993). Overall, only less than 30% of SHV enzymes which present amino acid substitutions related to extended-spectrum cephalosporin resistance (Chapter I, Table 1.7) have been biochemicaly characterized. However, these kinetic studies are of crucial importance.

A new IRS enzyme conferring increased resistance to amoxicillin plus clavulanic acid, SHV-72, was also characterized and, furthermore, the role of Lys234Arg substitution was investigated (Chapter IV). With the identification of this IRS enzyme, the number of natural occuring SHV enzymes that could confer inhibitor resistance was increased. Recently, another IRS enzyme was described with the same amino acid substitution plus the Leu35Gln substitution, SHV-56 (Dubois *et al.*, 2008).

The emergence of IRS enzymes raises the question of the possible appearance of CMS (Complex Mutant SHV) capable of conferring ESBL plus IRS phenotype. In fact, an enzyme was described in 1997, SHV-10, which possessed the ESBL-related amino acid substitutions of SHV-5 plus the IRS substitution, Ser130Gly (Prinarakis *et al*., 1997). This enzyme could not be considered a CMS, because the cephalosporinase and penicillinase activities associated with SHV-5 were reduced in SHV-10. This confirms that Ser130 is important in the interaction with cephalosporins and β-lactamase inhibitors (Jacob *et al.*, 1990).

The emergence of CMS by mutational events might be associated with a selection process within specific sites of infection. As most IRT and CMT enzymes have been recovered from urinary isolates, where penicillin-β-lactamase inhibitors reach higher

concentrations, the same could happen with complex mutant enzymes from SHV or even CTX-M families. The massive use of β-lactam plus β-lactamase inhibitor combinations in patients from the community and hospital environments also facilitates the appearance of these type of enzymes (Cantón *et al.*, 2008).

In Chapter V, where there was an investigation of extended- and broad-spectrum β-lactamase-producing *K. pneumoniae* isolated in 17 clinical health institutions, 18 new SHV enzymes were identified, of which 17 were non-ESBL. This study documents the substantial diversity detected among 207 *K. pneumoniae* strains collected in different regions of Portugal, where it was not possible to define an epidemic strain. Nevertheless, the genetic relatedness of 108 isolates studied by PFGE analysis identified a major profile type in our collection, 50% of which being GES-1 producers. Among the new SHV enzymes identified in the country, SHV-71, SHV-72, SHV-73 and SHV-80, all presenting an amino acid substitution, Ala146Val, described before in SHV-38 as contributing to an elevated carbapenem-hydrolytic activity (Poirel *et al.*, 2003). Could the presence of this amino acid substitution produce carbapenem-hydrolytic activity in these new SHV enzymes? According to the authors that described the SHV-38, the Ala146Val substitution is located at the N terminus of an alpha helix, neither in the β-lactam binding site nor in the catalytic site. The alpha helix where is located the amino acid Ala146 is parallel to positions 161 to 164 of the Ω loop at a distance of 3.1 Å, and the side chain, although hydrophobic, is oriented toward the solvent (Poirel *et al.*, 2003). However, the authors did not have data that explained how the amino acid substitution alters the behaviour of the SHV-38 enzyme towards imipenem. Nevertheless, the future characterization of this substitution in the four enzymes that we detected may increase the knowledge about carbapenem-hydrolysing SHV enzymes.

One of the other new bla_{SHV} genes we detected, $bla_{SHV\text{-}83}$, with a T2A mutation (Met5Lys), may be considered of particular interest, as one may wonder about a possible effect in the level of expression associated to this initiation codon. Under Ambler numbering, the Met at position 5 corresponds to the site of translation initiation (Ambler *et al.*, 1991). Other codons besides ATG, which codes for a Met, can be used to initiate translation (for example, GTG, which codes for a Val; and TTG, which codes for a Leu) (Lewin, 1994). In SHV-83, despite the fact that the mature protein is identical to SHV-1, the deduced translated sequence has an initial Lys residue; thus, either an unusual starting codon is being used or another Met residue in a different position is responsible for the beginning of translation. In fact, $bla_{OXA\text{-}20}$ translation initiates with a TTG (Leu) codon, which, according to the authors, implies a reduced level of expression (Naas *et al.*, 1998), this being the least efficient initiation codon in *E.coli* (Lewin, 1994). Recently, a SHV enzyme (SHV-104) that presented a Met5Leu substitution was also reported (http://www.lahey.org/studies).

Two other enzymes, SHV-106 and SHV-107, possess amino acid substitutions within the active site (Chapter V). The ESBL enzyme SHV-106 presents two amino acid substitutions, Tyr7Phe and Gly238Ser, the latter extensively studied and implicated in the increase of resistance to extended-spectrum cephalosporins (Huletsky *et al.*, 1993). However, SHV-107 showed a new amino acid substitution, Thr235Ala. In class A enzymes, the Thr235 residue is part of a conserved element (Lys234-Thr235-Gly236), situated on the β3 strand of a β-sheet in the α/β domain, forming the opposite wall of the catalytic cavity site (Delmas *et al.*, 2008). The structure and dynamics of other class A β-lactamases shows that the presence of Asp240Gly improves the interaction of residues Ser237 and Thr235 with ceftazidime. In SHV-107, the amino acid Thr is substituted by a smaller hydrophobic amino acid, Ala. Studies with site-directed mutagenesis produced a Ser235Ala substitution in a TEM-1 β-lactamase (Imtiaz *et al.*, 1993). In this study, by MIC determination, the *E. coli* strain bearing the mutant TEM-1 presented much lower values for cephalosporins than those of the *E. coli* expressing TEM-1 wild-type. Kinetic determinations showed similar results between the Ala235 mutant and TEM-1 wild-type for penicillins, but the cephalosporinase activity was highly reduced for the Ala235 mutant (Imtiaz *et al.*, 1993). Considering these results, one could suspect that Thr235Ala in SHV-107 may also influence the cephalosporinase activity. Nevertheless, future contributions of kinetic determinations of SHV-107 and eventually MDSs for the SHV-1 mutant carring the Ala235, will demonstrate the importance of this amino acid substitution in the resistance to penicillins and cephalosporins.

Despite the importance of ESBLs as a serious clinical problem for treatment of patients carrying ESBL-expressing *K. pneumoniae*, the clinical importance of the non-ESBL enzymes is yet to be revealed. Among these non-ESBL enzymes we identified SHV-71 and SHV-75 (Chapter V), which were also recently identified in *K. pneumoniae* isolates from Thailand (Kiratisin *et al.*, 2008). The authors demonstrated that these enzymes were co-expressed with CTX-M-14 and CTX-M-15, respectively. Co-expression of CTX-M enzymes and SHV penicillinases in *K. pneumoniae* strains is becoming very common (Kiratisin *et al.*, 2008; Romero *et al.*, 2005). However, co-expression of ESBL enzymes with other intrinsic *K. pneumoniae* β-lactamases, like OKP or LEN, is rarer (Hæggman *et al.*, 2004; Remeli *et al.*, 2007).

Until now, a reduced number of studies based on LEN and OKP β-lactamases have been published and only a few variants have been detected, when compared to other β-lactamase families, like TEM, SHV or CTX-M. In Chapter VI, the complete sequencing of 308 *K. pneumoniae bla* genes allowed for the identification and charaterization of 20 LEN and OKP encoding genes. Overall, 93.5% of our strains expressed SHV enzymes, thus belonging to group KpI. This is a higher proportion than previously reported for other collections (80 to

88%) (Brisse & Verhoef, 2001; Fu *et al.,* 2007). Groups KpII and KpIII were considerably less represented, with no more than 2.9% and 3.5% of the strains, respectively. These groups each contained 10% of the strains in another study (Brisse & Verhoef, 2001).

Studies based on the phylogeny of *bla* genes and on the phylogenies of the housekeeping genes *gyrA* and *mdh* suggest that the most probable origin of the *K. pneumoniae* β-lactamase families was diversification from a common ancestor, along side the evolutionary divergence of the phylogenetic groups (Hæggman *et al.*, 2004). More recently, the evolutionary divergence of bla_{OKP} subgroups (bla_{OKP-A} and bla_{OKP-B}) has been established and phylogenetic studies based on sequencing of *rpoB*, *gyrA*, and *mdh* genes showed a concordant subdivision of *K. pneumoniae* phylogenetic group KpII (Fèvre *et al.*, 2005). Overall, within the *K. pneumoniae* species, there has been an evolutionary divergence of chromosomal *bla* genes (Hæggman *et al.*, 2004).

In addition to the diversity referred to by Hæggman *et al.* (2004), we also document the high diversity of bla_{SHV} genes in Chapter VII; this observation allowed us to establish a classification system based on the synonymous mutations plus nonsynonymous mutation T92A. In general, among the 297 bla_{SHV} genes studied, 61 were identified as bla_{SHV-11} with 20 different nucleotide sequence frameworks and 59 were bla_{SHV-1} with 18 different nucleotide sequence frameworks. This study raised several important questions, such as what is the common ancestor of bla_{SHV-11} and bla_{SHV-1} derived genes or how long ago did these two parental genes diverged or even is there a parental gene more likely to evolve to a gene coding for an ESBL or IRS enzyme?

Although amino acid sequences evolve more slowly than DNA and are easier to align, studying protein evolution by modelling the evolutionary process on coding DNA is more appealing, because nucleotide sequences contain more information. Nucleotide synonymous mutations are always overlooked when performing evolutionary studies based on amino acid sequences. Phylogenetic studies that involve synonymous and non-synonymous mutations, as well as the presence or absence of mutations associated with antibiotic selective pressure – thus conferring ESBL or IRS phenotypes – are of high importance for the understanding of the evolution processes of SHV enzymes. However, the value of the phylogenetic information carried by nonsynonymous mutations, which are strongly selected, as ESBL and IRS genes evolved due to selective pressure to adapt quickly to their environment, may bias the overall results of the phylogenetic studies.

Nevertheless, to improve the outcome of this phylogenetic analysis, it would be of most importance to include all intrinsic β-lactamases expressed by *K. pneumoniae*. Other studies have already shown the phylogenetic relation between the SHV, LEN and OKP families, however, they did not account for the high diversity within these families (Hæggman

et al., 2004). Other evolutionary studies with SHV suggest that the ancestral SHV was already an ESBL, from which penicilinases, like SHV-1 and SHV-11, evolved by losing the ability to hydrolyse cephalosporins (Hall & Barlow, 2004). Nevertheless, it will be interesting to establish the common ancestor of SHV, LEN and OKP enzymes, as well as the evolutionary importance of the two SHV enzymes (SHV-1 and SHV-11) considered to be parental. Overall, our established classification system will be most usefull as a basis for evolutionary studies.

Another main issue we studied was the production of CTX-M enzymes by *E. coli* isolates. Among β-lactamase families, one of the most important is the rapidly spreading worldwide CTX-M family. These ESBLs present more than 80 variants subdivided into five major clusters, they derived from the chromosomal β-lactamase of *Kluyvera* spp., and are highly associated with mobile elements. In some geographical areas, CTX-M enzymes are already the most prevalent ESBL among *Enterobacteriaceae* (Rossolini *et al.*, 2008). A specific cluster associated with CTX-M-1, which has been shown to have the highest diversity, comprises one of the most disseminated β-lactamases – the CTX-M-15 enzyme. The gene coding for this enzyme is frequently located downstream of an IS*Ecp1* mobility element which, according to some studies, not only confers mobility to bla_{CTX-M} genes, but also probably provides the promoter for their expression (Cao *et al.*, 2002; Karim *et al.*, 2001; Poirel *et al.*, 2005).

As part of the worldwide dissemination, the CTX-M-15 enzymes were also detected in *E. coli* strains in Portugal, as stated in Chapter VIII. In this study, two genetically unrelated clinical strains from different geographical regions of the country, collected in 2004, were identified as producers of CTX-M-15, OXA-1 and TEM-1 enzymes. Although CTX-M-15 had already been detected in other *Enterobacteriaceae* in this country (Conceição *et al.*, 2005), it was the first time that the now common combination of CTX-M-15, OXA-1 and TEM-1 β-lactamases was described. As established at the time, it was urgent and essential to understand the real dissemination of CTX-M enzymes among Portuguese hospitals and community environments. Thus, in Chapter IX, a work to characterize the phenotype, genetic relatedness and β-lactamase produced by CTX-M-producing *E. coli* isolates identified in nine hospitals was embraced.

Overall, this surveillance study detected more than 65% of CTX-M enzymes among 181 ESBL-producing *E. coli* isolates, which is similar to the high rates showed by other surveillance studies (Rossolini *et al.*, 2008). However, more recent studies have showed even higher rates, like 100% in Thailand and 92% in Sweden (Fang *et al.*, 2008; Kiratisin *et al.*, 2008). These higher rates of CTX-M among total ESBLs enzymes, are most probably associated with high mobilization of the encoding genes. Barlow *et al.* (2008) found that the

bla$_{CTX-M}$ genes have been mobilized to plasmids almost 10 times more frequently than other class A β-lactamases. For example, IS*CR1* associated with CTX-M-2 and CTX-M-9 groups and IS*Ecp1* associated with CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 groups, are both known to contribute to this mobilization process (Poirel *et al.*, 2005; Rossolini *et al.*, 2008; Toleman *et al.*, 2006). Responsibilities were attributed to both elements for four of eight mobilizations from the chromosome of *Kluyvera* spp. to plasmids, as stated by Barlow *et al.* (2008), although IS*CR1* has been also reported to be responsible for mobilization of other *bla* genes (Naas *et al.*, 2006).

Intra- and inter-species horizontal spread of *bla*$_{CTX-M}$ genes is largely promoted by plasmids that may carry other resistance determinants (Bonnet, 2004). In fact, the percentage of bacterial strains that contained plasmids in the pre-antibiotic drug era and at the present, although error-prone, rose from 19% to 58-100% (Barlow *et al.*, 2008).

In our study, high level and high percentage of resistance to non-β-lactam antibiotics were also present. More than 93% of strains were resistant to fluoroquinolones, 88% were resistant to aminoglycosides and 26% were resistant to trimethoprim-sulfamethoxazole. Even though other resistance mechanisms besides β-lactamase production were not studied, plasmid-mediated fluoroquinolone resistance determinants, like QNR and/or AAC(6')-Ib-cr (aminoglycoside 6'-N-acetyltransferase type Ib-cr) variant, conferring co-resistance to aminoglycosides and to certain fluoroquinolones, are likely to be present. In fact, the plasmid association of *bla*$_{CTX-M}$ genes with these two recently identified types of plasmid-mediated resistance mechanisms has become quite common (Boyd *et al.*, 2004; Leflon-Guibout *et al.*, 2004; Machado *et al.*, 2007; Oteo *et al.*, 2006). Another association detected in this work, but in this case among *bla* genes, could be responsible for the lack of resistance of CTX-M producing strains towards β-lactamase inhibitors, until now undetected. The co-expression of *bla*$_{OXA-1}$ genes by 93% of strains possessing *bla*$_{CTX-M}$ genes implied a 26% of reduced susceptibility to amoxicillin plus clavulanic acid combination and 12% to piperacillin plus tazobactam combination. Could the co-expression of both these types of enzymes enable the emergence of CTX-M β-lactamases capable of conferring resistance to β-lactamase inhibitors?

Site-directed mutagenesis studies tried to establish the role of residue Arg276 of CTX-M-type enzymes, which is equivalent to the Arg244 of TEM enzymes, associated to the IRT phenotypes (Delmas *et al.*, 2006; Pérez-Llarena *et al.*, 2008). However, none of the studies could show an increase in MIC values towards β-lactam/β-lactamase-inhibitor combinations associated with substitutions of residue Arg276 and, furthermore, mutants showed decreased MIC values to cefotaxime as well as reduced catalytic efficiency to this antibiotic.

Overall, all CTX-M-producing strains were susceptible to carbapenems. However, the emergence of different types of carbapenemases could compromise the usefulness of this class of antibiotics (Walther-Rasmussen & Høiby, 2006; Walsh *et al.*, 2005). Nowadays, other types of antibiotics, like fosfomycin, nitrofurantoin and tigecycline, could be valid alternatives to carbapenems by reducing the selection pressure for ESBL-producing organisms (Garau, 2008). Nevertheless, none of these alternatives present total efficacy against ESBL producers and, so, improving antibiotics of current use and discovering new anti-Gram-negative drugs continues to be an important challenge.

Another important aspect of this work was the detection of dissemination of CTX-M producers within a specific hospital (Chapter IX, Table 9.6). Although, CTX-M-producing strains have been detected all over the country in nosocomial and community environments, the dissemination through the majority of wards in this hospital should raise questions of control infections. Classic control measures should be applied, such as avoiding all antibiotics to which the spreading strains or plasmid donors are resistant (thus reducing the selection pressure), as well as decreasing the use of procedures that are related to the risk of infection, and adopting adequate procedures of hand hygiene among clinical staff. Studies have shown that these types of interventions are important to infection control, both in nosocomial or community environments (Warren *et al.*, 2008).

Similar results were obtained in Chapter X, where the phenotypic and genetic profiles of 16 clinical *E. coli* ESBL producers isolated in an Algerian hospital were investigated. In this study, only CTX-M enzymes from group 1 were disseminated in different hospital wards, but no major epidemic strain was identified, in contrast to the Portuguese situation. Overall, more recent studies have also identified the same type of CTX-M enzymes disseminated among other *Enterobacteriaceae* in this country (Iabadene *et al.* 2008; Messai *et al.*, 2008; Touati *et al.*, 2008). The worldwide dissemination of CTX-M β-lactamases should be faced as a very serious problem, because these enzymes are now the most prevalent ESBL founded in clinical *E. coli* and *K. pneumoniae* isolates (Rossolini *et al.*, 2008).

Overall, we detected a high diversity of β-lactamases among clinical isolates of *E. coli* and *K. pneumoniae*, namely SHV, GES, LEN, OKP, CTX-M, OXA and TEM enzymes. The issues studied in this dissertation are of most importance to the comprehension of the general situation of β-lactamases and resistance in Portugal and constitute a contribution to the worldwide scenario. Nevertheless, some questions arose from this work that remain to be clarified and would constitute an interesting subject for future investigations.

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